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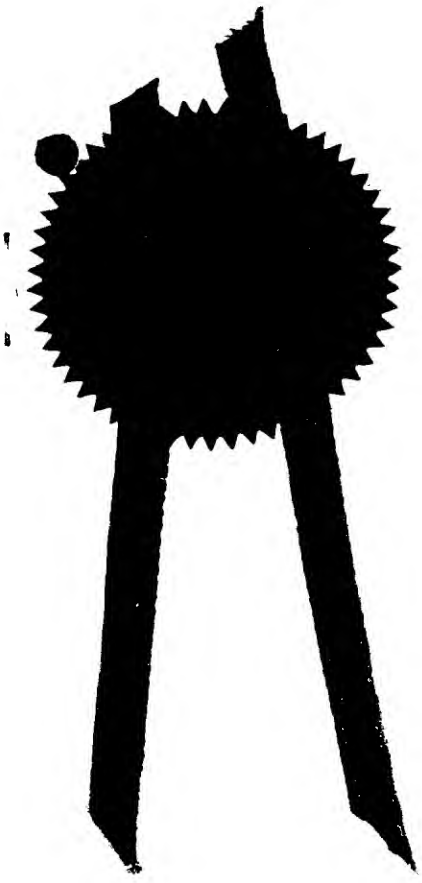
Date of Filing	:	18 FEBRUARY 1999
Application number	:	9900811-2
Applicants	:	NATIONAL UNIVERSITY OF SINGAPORE
Title of Invention	:	CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

I further certify that the annexed documents are not, as yet, open to public inspection.



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Singapore

20 August 1999

9900811-2

PATENTS FORM 1 *SECOND SCHEDULE* *Rule 19*

SINGAPORE
THE PATENTS ACT
(CHAPTER 221)
THE PATENTS RULES

The Registrar of Patents
Registry of Patents

REQUEST FOR THE GRANT OF A PATENT

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION.

I. Title of Invention	Chimeric gene constructs for generation of fluorescent transgenic ornamental fish		
II. Applicant(s) *(See note 2)*	*(a) Name*	National University of Singapore	
	Body Description/ Residency	Incorporated in Singapore	
	Street Name & Number	Industry & Technology Relations Office National University of Singapore 10 Kent Ridge Crescent	
	City	Singapore 119260	
	State	Singapore	
	Country	Singapore	
	(b) Name		
	Body Description/ Residency		
	Street Name & Number		
	City		
	State		
	Country		
	(c) Name		
	Body Description/		
	Residency *Street Name & Number*		
	City		
	State		
	Country		
III. Declaration of priority *(See note 3)*	*Country/Country Designated*		
	Filing Date		
	Country/Country Designated		*File no.*
	Filing Date		
	Country/Country Designated		*File no.*
	Filing Date		

SECOND SCHEDULE - continued

IV. Inventors (See note 4) (a) The applicant(s) is/are the sole/joint inventor(s). (b) A statement on Patents Form 8 is/will be furnished.				
	[ ] Yes	[X] No		
	[X] Yes	[ ] No		
V. Name of Agent (if any) (See note 5)	Applied Research Corporation			
VI. Address for Service (See note 6)	Block/Hse No		Level No	
	Unit No/PO Box	Kent Ridge P.O.BOX 1016	Postal Code	911101
	Street Name			
	Building Name			
VII. Claiming an earlier filing date under section 20(3), 26(6) or 47(4). (See note 7)	Application No			
	Filing Date			
VIII. Invention has been displayed at an International Exhibition (See note 8)	[ ] Yes	[X] No		
XI. Section 114 requirement (See note 9)	The invention relates to and/or uses a micro-organism deposited for the purposes of disclosure in accordance with section 114 with a depositary authority under the Budapest Treaty.			
	[ ] Yes	[X] No		

X. Check List (To be filled in by applicant or agent)

A. The application contains the following number of sheet(s):-

1. Request.	3	sheets
2. Description.	48	sheets
3. Claim(s).	2	sheets
4. Drawing(s).	9	sheets
5. Abstract.	1	sheets

B. The application as filed is accompanied by:-

1. Priority document.	
2. Translation of priority document.	
3. Statement of Inventorship & right to grant.	X
4. International Exhibition Certificate..	

XI. Signature(s) (See note 10)

Applicant (a)	
Date	18/2/99
Applicant (b)	
Date	
Applicant (c)	
Date	

SECOND SCHEDULE — *continued*

NOTES:

1. This form when completed, should be brought or sent to the Registry of Patents together with the prescribed fee and 3 copies of the description of the invention, and of any drawings.

2. Enter the name and address of each applicant in the spaces provided at paragraph II. Names of individuals should be indicated in full and the surname or family name should be underlined. The names of all partners in a firm must be given in full. The place of residence of each individual should also be furnished in the space provided. Bodies corporate should be designated by their corporate name and country of incorporation and, where appropriate, the state of incorporation within that country should be entered where provided. Where more than three applicants are to be named, the names and address of the fourth and any further applicants should be given on a separate sheet attached to this Form together with the signature of each of these further applicants.

3. The declaration of priority at paragraph III should state the date of the previous filing, the country in which it was made, and indicate the file number, if available. Where the application relied upon in an International Application or a regional patent application e.g. European patent application, one of the countries designated in that application (being one falling under the Patents (Convention Countries) Order) should be identified and the name of that country should be entered in the space provided.

4. Where the applicant or applicants is/are the sole inventor or the joint inventors, paragraph IV should be completed by marking the "YES" Box in the declaration (a) and the "NO" Box in the alternative statement (b). Where this is not the case, the "NO" Box in declaration (a) should be marked and a statement will be required to be filed on Patents Form 8.

5. If the applicant has appointed an agent to act on his behalf, the agent's name should be indicated in the spaces available at paragraph V.

6. An address for service in Singapore to which all documents may be sent must be stated at paragraph VI. It is recommended that a telephone number be provided if an agent is not appointed.

7. When an application is made by virtue of section 20(3), 26(6) or 47(4), the appropriate section should be identified at paragraph VII and the number of the earlier application or any patent granted thereon identified.

8. Where the applicant wishes an earlier disclosure of the invention by him at an International Exhibition to be disregarded in accordance with section 14(4)(c), then the "YES" box at paragraph VIII should be marked. Otherwise the "NO" box should be marked.

9. Where in disclosing the invention the application refers to one or more micro-organisms deposited with a depositary authority under the Budapest Treaty, then the "YES" box at paragraph IX should be marked. Otherwise the "NO" box should be marked.

10. Attention is drawn to rules 90 and 105 of the Patent Rules. Where there are more than three applicants, see also Note 2 above.

11. Applicants resident in Singapore are reminded that if the Registry of Patents considers that an application contains information the publication of which might be prejudicial to the defence of Singapore or the safety of the public, it may prohibit or restrict its publication or communication. Any person resident in Singapore and wishing to apply for patent protection in other countries must first obtain permission from the Singapore Registry of Patents unless they have already applied for a patent for the same invention in Singapore. In the latter case, no application should be made overseas until at least two months after the application has been filed in Singapore.

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Application Filing Date: / /
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CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

FIELD OF THE INVENTION

This invention relates to fish gene promoters and chimeric gene constructs with these promoters for generation of transgenic fish, particularly fluorescent transgenic ornamental fish.

BACKGROUND OF THE INVENTION

Transgenic technique involves the transfer of a foreign gene into a host organism enabling the host to acquire a new and inheritable trait. The technique was first developed in mice by Gordon et al. (1980). They injected foreign DNA into fertilized eggs and found that some of the mice developed from the injected eggs retained the foreign DNA. Applying the same technique, Palmiter et al. (1982) have introduced a chimeric gene containing a rat growth hormone gene under a mouse heavy metal inducible gene promoter and generated the first batch of genetically engineered supermice, which are almost twice as large as non-transgenic siblings. This work has opened a promising avenue in using the transgenic approach to render animals new and beneficial traits for livestock husbandry and aquaculture.

In addition to the stimulation of somatic growth for increasing the gross production of animal husbandry and aquaculture, the transgenic technique also has many other potential applications. First of all, transgenic animals can be used as a bioreactor to produce commercially useful compounds by expression of a useful foreign gene in milk or in blood. Many pharmaceutically useful protein factors have been expressed in this way. For example, the human α1-antitrypsin, which is commonly used to treat emphysema, has been expressed at a concentration as high as 35 mg/ml (10% of milk proteins) in the milk of transgenic sheep (Wright et al., 1991). Similarly, the transgenic technique can also be used to improve the nutritional value of milk by selectively increasing the levels of certain valuable proteins such as caseins and by supplementing certain new and useful proteins such as lysozyme for antimicrobial activity (Maga and Murray, 1995). Second, transgenic mice have been widely used in medical research, particularly in the generation of transgenic animal models for human disease studies (Lathe and Mullins, 1993). More recently, it has been proposed to use transgenic pigs as organ donors for xenotransplantation by expressing human regulators of complement activation to prevent hyperacute rejection during organ transplantation (Cozzi and White, 1995). The

development of disease resistant animals has also been tested in transgenic mice (e.g. Chen et al., 1988).

Fish are also an intensive research subject in the transgenic study. There are many ways of introducing a foreign gene into fish, including microinjection (e.g. Zhu et al., 1985; Du et al., 1992), electroporation (Powers et al., 1992), sperm-mediated gene transfer (Khoo et al., 1992; Sin et al., 1993), gene bombardment or gene gun (Zelemin et al., 1991), and liposome-mediated gene transfer (Szelei et al., 1994). The first transgenic fish report was published by Zhu et al. (1985) using a chimeric gene construct consisting of a mouse metallothionein gene promoter and a human growth hormone gene. Most of the early transgenic fish studies have concentrated on growth hormone gene transfer with an aim at generating fast growing "superfish". A majority of early attempts used heterologous growth hormone genes and promoters and failed to produce gigantic superfish (e.g. Chourrout et al., 1986; Penman et al., 1990; Brem et al., 1988; Gross et al., 1992). But enhanced growth of transgenic fish has been demonstrated in several fish species including Atlantic salmon, several species of Pacific salmons, and loach (e.g. Du et al., 1992; Delvin et al., 1994, 1995; Tsai et al., 1995).

The zebrafish, *Danio rerio,* is a new model organism for vertebrate developmental biology. As an experimental model, the zebrafish offers several major advantages such as easy availability of eggs and embryos, tissue clarity throughout the embryogenesis, external development, short generation time and easy maintenance of both the adult and the young. Transgenic zebrafish have been used as an experimental tool in zebrafish developmental biology. However, despite the fact that the first transgenic zebrafish was reported a decade ago (Stuart et al., 1988), most transgenic zebrafish work conducted so far used heterologous gene promoters or viral gene promoters: e.g. viral promoters from SV40 (simian virus 40) and RSV (Rous sarcoma virus) (Stuart et al., 1988, 1990; Bayer and Campos-Ortega, 1992), a carp actin promoter (Liu et al., 1990), and mouse homeobox gene promoters (Westerfield et al., 1992). As a result, the expression pattern of a transgene in many cases is variable and unpredictable.

GFP (green fluorescent protein) was isolated from a jelly fish, *Aqueous victoria.* The wild type GFP emits green fluorescence at a light length of 508 nm upon stimulation with ultraviolet light (395 nm). The primary structure of GFP has been elucidated by cloning of its cDNA and genomic DNA (Prasher et al., 1992). A modified GFP, also called EGFP for enhanced green fluorescent protein, has been generated artificially and it contains mutations that allow the protein to emit a stronger green light and its coding sequence has also been optimized for higher expression in mammalian cells based on preferable human codons. As a result, EGFP fluorescence is about 40 times stronger than

the wild type GFP in mammalian cells (Yang et al., 1996). GFP (including EGFP) has become a popular tool in cell biology and transgenic research. By fusing GFP with a tested protein, the GFP fusion protein can be used as an indicator of the subcellular location of the tested protein (Wang and Hazelrigg, 1994) . By transformation of cells with a functional GFP gene, the GFP can be used as a marker to identify expressing cells (Chalfie et al., 1994). Thus, the GFP gene has become an increasingly popular reporter gene for transgenic research as GFP can be easily detected by a non-invasive approach.

The GFP gene (including EGFP gene) has also been introduced into zebrafish in several previous reports by using various gene promoters, including Xenopus *elongation factor 1α* enhancer-promoter (Amsterdam et al., 1995, 1996), rat *myosin light-chain* enhancer (Moss et al., 1996), zebrafish *GATA-1* and *GATA-3* promoters (Meng et al., 1997; Long et al., 1997), zebrafish *α-* and *β-actin* promoters (Higashijima et al., 1997), and tilapia *insulin-like growth factor 1* promoter (Chen et al., 1998). All of these transgenic works aim at either developing a GFP transgenic system for gene expression analysis or at testing regulatory DNA elements in gene promoters.

SUMMARY OF THE INVENTION

It is a primary objective of the invention to clone fish gene promoters of skin specificity, muscle specificity or ubiquitous function and to use these promoters to develop effective gene constructs for production of transgenic fish.

It is another objective of the invention to develop fluorescent transgenic ornamental fish using these gene constructs. By applying different gene promoters, tissue-specific or ubiquitous, to drive the GFP gene, GFP could be expressed in different tissues or ubiquitously. Thus, these transgenic fish may be skin fluorescent, muscle fluorescent, ubiquitously fluorescent, or inducibly fluorescent. These transgenic fish may be used for ornamental purposes, for monitoring environmental pollution, and for basic studies such as recapitulation of gene expression programs or monitoring cell lineage and cell migration. These transgenic fish may be used for cell transplantation and nuclear transplantation or fish cloning.

Other objectives, features and advantages of the present invention will become apparent from the detailed description which follows, or may be learned by practice of the invention.

Three zebrafish gene promoters of different characteristics were isolated and three chimeric gene constructs containing a zebrafish gene promoter and EGFP DNA were

made: pCK-EGFP, pMCK-EGFP and pARP-EGFP. The first chimeric gene construct, CK-EGFP, contains a zebrafish cytokeratin (CK) gene promoter (2.2 kb) which is specifically or predominantly expressed in skin epithelia. The second one, MCK-EGFP, contains a muscle-specific promoter (1.5 kb) from a zebrafish muscle creatine kinase (MCK) gene and the gene is only expressed in the muscle tissue. The third one, ARP-EGFP, contains a strong and ubiquitously expressed promoter from a zebrafish acidic ribosomal protein (ARP) gene. These three chimeric gene constructs have been introduced into zebrafish at the one cell stage by microinjection. In all cases the GFP expression patterns were consistent with the specificities of the promoters. GFP was predominantly expressed in skin epithelia with pCK-EGFP, specifically expressed in muscles with pMCK-EGFP, and ubiquitously expressed in all tissues with pARP-EGFP.

These chimeric genes will be useful to generate green fluorescent transgenic fish. The GFP transgenic fish emit green fluorescence light under a blue light and this feature makes the genetic engineered fish unique and attractive in the ornamental fish market. Meanwhile, the fluorescent transgenic fish are also useful as research models for embryonic studies such as cell lineage, cell migration, cell and nuclear transplantation etc.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1G are photographs showing expression of CK (Figs. 1A-1C), MCK (Figs. 1D-1E) and ARP (Figs. 1F-1G) mRNAs in zebrafish embryos as revealed by whole mount *in situ* hybridization (detailed description of the procedure can be found in Thisse et al., 1993). (Fig. 1A) A 28 hpf (hour postfertilization) embryo hybridized with a CK antisense riboprobe. (Fig. 1B) Enlargement of the mid-part of the embryo shown in Fig. 1A. (Fig. 1C) Cross-section of the embryo in Fig. 1A. (Fig. 1D) A 30 hpf embryo hybridized with an MCK antisense riboprobe. (Fig. 1E) Cross-section of the embryo in Fig 1D. (Fig. 1F) A 28 hpf embryo hybridized with an ARP antisense riboprobe. (Fig. 1G) Cross-section of the embryo in Fig. 1F. Arrows indicate the planes for cross-sections and box in panel A indicates the enlarged region shown in panel B.

Fig. 2 is a digitized image showing distribution of CK, MCK and ARP mRNAs in adult tissues. Total RNAs were prepared from selected adult tissues as indicated at the top of each lane and analyzed by Northern blot hybridization (detailed description of the procedure can be found in Gong et al., 1992). Three identical blots were made from the same set of RNAs and hybridized with the CK, MCK and ARP probes, respectively.

Fig. 3. is a schematic representation of the strategy of promoter cloning. Restriction enzyme digested genomic DNA was ligated with a short linker DNA which consists of

Oligo 1 and Oligo 2. Nested PCR reactions were then performed: the first round PCR used linker specific primer L1 and gene specific primers G1, where G1 is CK1, MCK1 or ARP1 in the described embodiments, and the second round linker specific primer L2 and gene specific primer G2, where G2 is CK2, MCK2 or ARP2, respectively in the described embodiments.

Fig. 4 is a schematic map of the chimeric gene construct, pCK-EGFP. The 2.2 kb zebrafish CK promoter region is inserted into the pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish CK promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pCK-EGFP is 6.4 kb.

Fig. 5 is a schematic map of the chimeric gene construct, pMCK-EGFP. The 1.5 kb zebrafish MCK promoter region is inserted into the pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish MCK promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pMCK-EGFP is 5.7 kb.

Fig. 6 is a schematic map of the chimeric gene construct, pARP-EGFP. The 2.2 kb zebrafish ARP promoter/1st intron region is inserted into the pEGFP-1 (Clonetech) at the EcoRI and BamHI site as indicated. In the resulting chimeric DNA construct, the EGFP gene is under control of the zebrafish ARP promoter. Also shown is the kanamycin/neomycin resistance gene (Kan^r/Neo^r) in the backbone of the original pEGFP-1 plasmid. The total length of the recombinant plasmid pARP-EGFP is 6.4 kb.

Fig. 7 is a photograph of a typical transgenic zebrafish fry (4 days old) with pCK-EGFP, which emits green fluorescence from skin epithelia under a blue light.

Fig. 8 is a photograph of a typical transgenic zebrafish fry (3 days old) with pMCK-EGFP, which emits green fluorescence from skeletal muscles under a blue light.

Fig. 9 is a photograph of a typical transgenic zebrafish fry (2 days old) with pARP-EGFP, which emits green fluorescence under a blue light from a variety of cell types such as skin epithelia, muscle cells, lens, neural tissues, notochord, circulating blood cells and yolk cells.

DETAILED DESCRIPTION OF THE INVENTION

Gene Constructs

To develop successful transgenic fish with a predictable pattern of transgene expression, the first step is to make a gene construct suitable for transgenic studies. The gene construct generally consists of three portions: a gene promoter, a structural gene and transcriptional termination signals. The gene promoter would determine where, when and under what conditions the structural gene is turned on. The structural gene contains a protein coding region that would determine the protein to be synthesized and thus the biological function. The transcription termination signals consist of two parts: a polyadenylation signal and a transcriptional termination signal after the polyadenylation signal. Both are important to terminate the gene transcription. Among the three portions, selection of a promoter is very important for successful transgenic study, and it is preferable to use a homologous promoter (homologous to the host fish) to ensure accurate gene activation in the transgenic host.

Recombinant DNA Constructs

Recombinant DNA constructs comprising one or more of the DNA or RNA sequences described herein and an additional DNA and/or RNA sequence are also included within the scope of this invention. These recombinant DNA constructs usually have sequences which do not occur in nature or exist in a form that does not occur in nature or exist in association with other materials that do not occur in nature. The DNA and/or RNA sequences described hereinabove are "operably linked" with other DNA and/or RNA sequences. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as part of a preprotein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the coding sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous (or in close proximity to) and, in the case of secretory leaders, contiguous and in reading phase.

The sequences of some of the DNAs, and the corresponding proteins encoded by the DNA, which are useful in the invention are set forth in the attached Sequence Listing.

The complete cytokeratin (CK) cDNA sequence is shown in SEQ ID NO:1, and its deduced amino acid sequence is shown in SEQ ID NO:2. The binding sites of the gene specific primers for promoter amplification, CK1 and CK2, are indicated. The extra

nucleotides introduced into CK2 for generation of a restriction site are shown as a misc_feature in the primer sequence SEQ ID NO:11. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:1.

The complete muscle creatine kinase (MCK) cDNA sequence is shown in SEQ ID NO:3, and its deduced amino acid sequence is shown in SEQ ID NO:4. The binding sites of the gene specific primers for promoter amplification, MCK1 and MCK2, are indicated. The extra nucleotides introduced into MCK1 and MCK2 for generation of restriction sites are shown as a misc_feature in the primer sequences SEQ ID NOS:12 and 13, respectively. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:3.

The complete acidic ribosomal protein P0 (ARP) cDNA sequence is shown in SEQ ID NO:5, and its deduced amino acid sequence is shown in SEQ ID NO:6. The binding sites of the gene specific primers for promoter amplification, ARP1 and ARP2, are indicated. The extra nucleotides introduced to ARP2 for generation of a restriction site are shown as a misc_feature in the primer sequence SEQ ID NO:15. A potential polyadenylation signal, AATAAA, is indicated in SEQ ID NO:5.

SEQ ID NO:7 shows the complete sequence of the 2.2 kb CK promoter region. A putative TATA box is shown, and the 3' nucleotides identical to the 5' CK cDNA sequence are shown as a misc_feature. The binding site of the second gene specific primer, CK2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:11.

SEQ ID NO:8 shows the complete sequence of the 1.5 kb MCK promoter region. A putative TATA box is shown, and the 3' nucleotides identical to the 5' MCK cDNA sequence are shown as a misc_feature in SEQ ID NO:8. The binding site of the second gene specific primer, MCK2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:13.

SEQ ID NO:9 shows the complete sequence of the 2.2 kb ARP promoter region including the first intron. The first intron is shown, and the 3' nucleotides identical to the 5' ARP cDNA sequence are shown as misc_features. No typical TATA box is found. The binding site of the second gene specific primer, ARP2, is shown. The introduced BamHI site is indicated as a misc_feature in the primer sequence SEQ ID NO:15.

Specifically Exemplified Polypeptides/DNA

The present invention contemplates use of DNA that codes for various polypeptides and other types of DNA to prepare the gene constructs of the present invention. DNA that codes for structural proteins, such as fluorescent peptides including GFP, EGFP, BFP,

EBFP, YFP, EYFP, CFP, ECFP and enzymes such as luciferase, ß-galactosidase; chloramphenicol acetyltransferase, etc. are useful in the present invention. More particularly, the DNA may code for polypeptides comprising the sequences exemplified in SEQ ID NOS:2, 4 and 6. The present invention also contemplates use of particular DNA sequences, including regulatory sequences, such as promoter sequences shown in SEQ ID NOS: 7, 8 and 9. Finally, the present invention also contemplates the use of additional DNA sequences, described generally herein or described in the references cited herein, for various purposes.

Chimeric Genes

The present invention also encompasses chimeric genes comprising a promoter described herein operatively linked to a heterologous gene. Thus, a chimeric gene can comprise a promoter of a zebrafish operatively linked to a zebrafish structural gene other than that normally found linked to the promoter in the genome. Alternatively, the promoter can be operatively linked to a gene that is exogenous to a zebrafish, as exemplified by the GFP and other genes specifically exemplified herein. Furthermore, a chimeric gene can comprise an exogenous promoter linked to any structural gene not normally linked to that promoter in the genome of an organism.

Variants of Specifically Exemplified Polypeptide

DNA which codes for variants of the specifically exemplified polypeptides are also encompassed by the present invention. Possible variants include allelic variants and corresponding polypeptides from other organisms, particularly other organisms of the same species, genus or family. The variants may have substantially the same characteristics as the natural polypeptides. The variant polypeptide will possess the primary property of concern for the polypeptide. For example, the polypeptide will possess one or more or all of the primary physical (e.g., color) and/or biological (e.g., enzymatic activity) properties of the specifically described polypeptide. DNA of the structural genes of the present invention will encode a protein that produces a fluorescent or chemiluminescent light under conditions appropriate to the particular polypeptide in one or more tissues of a fish. Preferred tissues for expression are skin, muscle, eye and bone.

Substitutions, Additions and Deletions

As possible variants of the above specifically exemplified polypeptides, the polypeptide may have additional individual amino acids or amino acid sequences inserted into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof so long as the polypeptide possesses the desired physical and/or biological

characteristics. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide so long as the polypeptide possesses the desired physical characteristics. Amino acid substitutions may also be made in the sequences so long as the polypeptide possesses the desired physical and biochemical characteristics. DNA coding for these variants can be used to prepare gene constructs of the present invention.

Sequence Identity at the Amino Acid Level

The variants of polypeptides contemplated herein should possess more than 75% sequence identity (sometimes referred to as homology), preferably more than 85% identity, most preferably more than 95% identity, even more preferably more than 98% identity to the naturally occurring and/or specifically exemplified polypeptides or fragments thereof described herein. To determine this homology, two polypeptides are aligned so as to obtain a maximum match using gaps and inserts.

Two sequences are said to be "identical" if the sequence of residues is the same when aligned for maximum correspondence as described below. The term "complementary" applies to nucleic acid sequences and is used herein to mean that the sequence is complementary to all or a portion of a reference polynucleotide sequence.

Optimal alignment of sequences for comparison can be conducted by the local homology algorithm of Smith and Waterman (1981), by the homology alignment method of Needleman and Wunsch (1970), by the search for similarity method of Pearson and Lippman (1988), or the like. Computer implementations of the above algorithms are known as part of the Genetics Computer Group (GCG) Wisconsin Genetics Software Package (GAP, BESTFIT, BLASTA, FASTA and TFASTA), 575 Science Drive, Madison, WI.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.* "gaps") as compared to the reference sequence for optimal alignment of the two sequences being compared. The percentage identity is calculated by determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window and multiplying the result by 100 to yield the percentage of sequence identity. Total identity is then determined as the average identity over all of the windows that cover the complete query sequence.

Fragments of Polypeptide

Genes which code for fragments of the full length polypeptides such as proteolytic cleavage fragments which contain at least one, and preferably all, of the above-listed physical and/or biological properties are also encompassed by the present invention.

DNA and RNA

The invention encompasses DNA that codes for any one of the above-described polypeptides including, but not limited to, those shown in SEQ ID NOS:2, 4, and 6, including fusion polypeptides, variants and fragments thereof. The sequence of certain particularly useful cDNAs which encode polypeptides are shown in SEQ ID NOS:1, 3 and 5. The present invention also includes cDNA as well as genomic DNA containing or comprising the requisite nucleotide sequences as well as corresponding RNA and antisense sequences.

Cloned DNA within the scope of the invention also includes allelic variants of the specific sequences presented in the attached Sequence Listing. An "allelic variant" is a sequence that is a variant from that of the exemplified nucleotide sequence, but represents the same chromosomal locus in the organism. In addition to those which occur by normal genetic variation in a population and perhaps fixed in the population by standard breeding methods, allelic variants can be produced by genetic engineering methods. A preferred allelic variant is one that is found in a naturally occurring organism, including a laboratory strain. Allelic variants are either silent or expressed. A silent allele is one that does not affect the phenotype of the organism. An expressed allele results in a detectable change in the phenotype of the trait represented by the locus.

A nucleic acid sequence "encodes" or "codes for" a polypeptide if it directs the expression of the polypeptide referred to. The nucleic acid can be DNA or RNA. Unless otherwise specified, a nucleic acid sequence that encodes a polypeptide includes both the transcribed strand and the mRNA or the DNA representative of the mRNA. An "antisense" nucleic acid is one that is complementary to all or part of a strand representative of mRNA, including untranslated portions thereof.

Degenerate Sequences

In accordance with degeneracy of genetic code, it is possible to substitute at least one base of the base sequence of a gene by another kind of base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, the DNA

of the present invention may also have any base sequence that has been changed by substitution in accordance with degeneracy of genetic code.

DNA Modification

The DNA is readily modified by substitution, deletion or insertion of nucleotides, thereby resulting in novel DNA sequences encoding the polypeptide or its derivatives. These modified sequences are used to produce mutant polypeptide and to directly express the polypeptide. Methods for saturating a particular DNA sequence with random mutations and also for making specific site directed mutations are known in the art; see *e.g.* Sambrook et al *(1989)*..

Hybridizable Variants

The DNA molecules useful in accordance with the present invention can comprise a nucleotide sequence selected from the group consisting of SEQ ID NOS.:1, 3, 5 and 7-19, or can comprise a nucleotide sequence that hybridizes to a DNA molecule comprising the nucleotide sequence of SEQ ID NOS.:1, 3 or 5 under salt and temperature conditions providing stringency at least as high as that equivalent to 5x SSC and 42°C and that codes on expression for a polypeptide that has one or more or all of the above-described physical and/or biological properties. The present invention also includes polypeptides coded for by these hybridizable variants. The relationship of stringency to hybridization and wash conditions and other considerations of hybridization can be found in Chapters 11 and 12 of Sambrook et al (1989). The present invention also encompasses functional promoters which hybridize to SEQ ID NOS:7, 8 or 9 under the above-described conditions. DNA molecules of the invention will preferably hybridize to reference sequences under more stringent conditions allowing the degree of mismatch represented by the degrees of sequence identity enumerated above. The present invention also encompasses functional primers or linker oligonucleotides set forth in SEQ ID NOS:10-19 or larger primers comprising these sequences, or sequences which hybridize with these sequences under the above-described conditions. The primers usually have a length of 10-50 nucleotides, preferably 15-35 nucleotides, more preferably 18-30 nucleotides.

Vectors

The invention is further directed to a replicable vector containing cDNA that codes for the polypeptide and that is capable of expressing the polypeptide.

The present invention is also directed to a vector comprising a replicable vector and a DNA sequence corresponding to the above described gene inserted into said vector. The

vector may be an integrating or non-integrating vector depending on its intended use and is conveniently a plasmid.

Transformed Cells

The invention further relates to a transformed cell or microorganism containing cDNA or a vector which codes for the polypeptide or a fragment or variant thereof and that is capable of expressing the polypeptide.

Expression Systems Using Vertebrate Cells

Interest has been great in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of vertebrate host cell lines useful in the present invention preferably include cells from any of the fish described herein. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing genomic DNA is used or if an intron is necessary to optimize expression of a cDNA), a polyadenylation site, and a transcription termination sequence.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

Example I: Isolation of skin-specific, muscle-specific and ubiquitously expressed zebrafish cDNA clones.

cDNA clones were isolated and sequenced as described by Gong et al. (1997). Basically, random cDNA clones were selected from zebrafish embryonic and adult cDNA libraries and each clone was sequenced partially by a single sequencing reaction. The partial sequences were then used to identify the sequenced clones for potential function and tissue specificity. Out of 261 distinct clones identified by this approach, three of them were selected for skin specificity (clone A39 encoding cytokeratin, CK), for muscle specificity (clone E146 encoding muscle creatine kinase, MCK), and for ubiquitous expression (clone A150 encoding acidic ribosomal protein P0, ARP), respectively.

The three cDNA clones were then sequenced completely and their complete cDNA sequences with deduced amino acid sequences are shown in SEQ ID NOS:1, 3 and 5, respectively. A39 encodes a type II basic cytokeratin and its closest homolog in mammals

is cytokeratin 8 (65-68% amino acid identity). E146 codes for the zebrafish MCK and its amino acid sequence shares ~87% identity with mammalian MCKs. The amino acid sequence of zebrafish ARP deduced from the A150 clone is 87-89% identical to those of mammalian ARPs.

To demonstrate their expression patterns, whole mount *in situ* hybridization was carried out for developing embryos and Northern blot analyses were carried out for selected adult tissues and for developing embryos.

As indicated by whole mount *in situ* hybridization, cytokeratin mRNA was specifically expressed in the embryonic surface (Figs. 1A-1C) and cross section of *in situ* hybridized embryos confirmed that the expression was only in skin epithelia (Fig. 1C). Ontogenetically, the cytokeratin mRNA appeared before 4 hpf and it is likely that the transcription of the cytokeratin gene starts at mid-blastula transition when the zygotic genome is activated. By *in situ* hybridization, a clear cytokeratin mRNA signal was detected in highly flattened cells of the superficial layer in blastula and the expression remained in the superficial layer which eventually developed into skin epithelia including the yolk sac. In adult tissues, cytokeratin mRNA was predominantly detected in the skin and also weakly in several other tissues including the eye, gill, intestine and muscle, but not in the liver and ovary (Fig. 2). Therefore, the cytokeratin mRNA is predominantly, if not specifically, expressed in skin cells.

MCK mRNA was first detected in the first few anterior somites in 10 somite stage embryos (14 hpf) and at later stages the expression is specifically in skeletal muscle (Fig. 1D) and in heart (data not shown). When the stained embryos are cross-sectioned, the MCK mRNA signal was found exclusively in the trunk skeletal muscles (Fig. 1E). In adult tissues, MCK mRNA was detected exclusively in the skeletal muscle (Fig. 2).

ARP mRNA was expressed ubiquitously and it is presumably a maternal mRNA since it is present in the ovary as well as in embryos at one cell stage. In *in situ* hybridization experiments, an intense hybridization signal was detected in most tissues. An example of a hybridized embryo at 28 hpf is shown in Fig. 1F. In adults, ARP mRNA was abundantly expressed in all tissues examined except for the brain where a relatively weak signal was detected (Fig. 2). These observations confirmed that the ARP mRNA is expressed ubiquitously.

Example II: Isolation of zebrafish gene promoters

Three zebrafish gene promoters were isolated by a linker-mediated PCR method as described by Liao *et al.*, (1997) and as exemplified by the diagrams in Fig. 3. The whole

procedure includes the following steps: 1) designing of gene specific primers; 2) isolation of zebrafish genomic DNA; 3) digestion of genomic DNA by a restriction enzyme; 4) ligation of a short linker DNA to the digested genomic DNA; 5) PCR amplification of the promoter region; and 6) DNA sequencing to confirm the cloned DNA fragment. The following is the detailed description of these steps.

1. Designing of gene specific primers

Gene specific PCR primers were designed based on the 5' end of the three cDNA sequences and the region used for the sequences are shown in Figs. 1-3.

The two cytokeratin gene specific primers are:

CK1 (SEQ ID NO:10)

CK2 (SEQ ID NO:11), where the first six nucleotides are for creation of an EcoRI site to facilitate cloning.

The two muscle creatine kinase gene specific primers are:

MCK1 (SEQ ID NO:12), where the first five nucleotides are for creation of an EcoRI site to facilitate cloning.

MCK2 (SEQ ID NO:13), where the first three nucleotides are for creation of an EcoRI site to facilitate cloning.

The two acidic ribosomal protein P0 gene specific primers are:

ARP1 (SEQ ID NO:14)

ARP2 (SEQ ID NO:15), where the first six nucleotides are for creation of an EcoRI site to facilitate cloning.

2. Isolation of zebrafish genomic DNA

Genomic DNA was isolated from a single individual fish by a standard method (Sambrook *et al.*, 1989). Generally, an adult fish was quickly frozen in liquid nitrogen and ground into powder. The ground tissue was then transferred to an extraction buffer (10 mM Tris, pH 8, 0.1 M EDTA, 20 μg/ml RNase A and 0.5% SDS) and incubated at 37°C for 1 hour. Proteinase K was added to a final concentration of 100 μg/ml and gently mixed until

the mixture appeared viscous, followed by incubation at 50°C for 3 hours with periodical swirling. The genomic DNA was gently extracted three times by phenol equilibrated with Tris-HCl (pH 8), precipitated by adding 0.1 volume of 3 M NaOAc and 2.5 volumes of ethanol, and collected by swirling on a glass rod, then rinsed in 70% ethanol.

3. Digestion of genomic DNA by a restriction enzyme

Genomic DNA was digested with the selected restriction enzymes. Generally, 500 units of restriction enzyme were used to digest 50 μg of genomic DNA overnight at the optimal enzyme reaction temperature (usually at 37°C).

4. Ligation of a short linker DNA to the digested genomic DNA

The linker DNA was assembled by annealing equal moles of the two linker oligonucleotides, Oligo1 (SEQ ID NO:16) and Oligo 2 (SEQ ID NO:17). Oligo 2 was phosphorylated by T4 polynucleotide kinase prior to annealing. Restriction enzyme digested genomic DNA was filled-in or trimmed with T4 DNA polymerase, if necessary, and ligated with the linker DNA. Ligation was performed with 1 μg of digested genomic DNA and 0.5 μg of linker DNA in a 20 μl of reaction containing 10 units of T4 DNA ligase at 4°C overnight.

5. PCR amplification of promoter region

PCR was performed with Advantage Tth Polymerase Mix (Clontech). The first round of PCR was performed using a linker specific primer L1 (SEQ ID NO:18) and a gene specific primer G1 (CK1, MCK1 or ARP1). Each reaction (50 μl) contains 5 μl of 10x Tth PCR reaction buffer (1X= 15 mM KOAc, 40 mM Tris, pH 9.3), 2.2 μl of 25 mM $Mg(OAc)_2$, 5 μl of 2 mM dNTP, 1 μl of L1 (0.2 μg/μl), 1 μl of G1 (0.2 μg/μl) , 33.8 μl of H_2O, and 1 μl (50 ng) of linker ligated genomic DNA and 1 μl of 50x Tth polymerase mix (Clontech). The cycling conditions were as follows: 94°C/1 min, 35 cycles of 94°C/30 sec and 68°C/6 min, and finally 68°C/8 min. After the primary round of PCR was completed, the products were diluted 100 fold. One μl of diluted PCR product was used as template for the second round of PCR (nested PCR) with a second linker specific primer L2 (SEQ ID NO:19) and a second gene specific primer G2 (CK2, MCK2 or ARP2), as described for the primary PCR but with the following modification: 94°C/1 min, 25 cycles of 94°C/30 sec and 68°C/6 min, and finally 68°C/8 min. Both the primary and secondary PCR products were analyzed on a 1% agarose gel.

6. DNA sequencing to confirm the cloned DNA fragment

PCR products were purified from the agarose gel following electrophoresis and cloned into a TA vector, pT7Blue (Novogen). DNA sequencing was performed by dideoxynucleotide chain termination method using a T7 Sequencing Kit purchased from Pharmacia. Complete sequences of these promoter regions were obtained by automatic sequencing using a dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and an ABI 377 automatic sequencing machine.

The isolated cytokeratin gene promoter is 2.2 kb. In the 3' proximal region immediately upstream of a portion identical to the 3' part of the CK cDNA sequence, there is a putative TATA box perfectly matching to a consensus TATA box sequence. The 164 bp of the 3' region is identical to the 5' UTR (untranslated region) of the cytokeratin cDNA. Thus, the isolated fragment was indeed derived from the same gene as the cytokeratin cDNA clone (SEQ ID NO:7). Similarly, a 1.5 kb 5' flanking region was isolated from the muscle creatine kinase gene, a putative TATA box was also found in its 3' proximal region and the 3' region is identical to the 5' portion of the MCK cDNA clone (SEQ ID NO:8). A 2.2 kb fragment was amplified for the ARP gene. By alignment of its sequence with the ARP cDNA clone, we found a 1.3 kb intron in the 5' UTR (SEQ ID NO:9). As a result, the isolated ARP promoter is only about 0.8 kb long.

Example III: Generation of green fluorescent transgenic fish

The isolated zebrafish gene promoters were inserted into the plasmid pEGFP-1, which contains an EGFP structural gene whose codons have been optimized according to preferable human codons. All of the three promoter fragments were inserted into pEGFP-1 at the EcoRI and BamHI site and the resulting recombinant plasmids were named pCK-EGFP (Fig. 4), pMCK-EGFP (Fig. 5), and pARP-EGFP, respectively (Fig. 6).

Linearized plasmid DNAs at a concentration of 500 μg/ml (for pCK-EGFP and pMCK-EGFP) in 0.1 M Tris-HCl (pH 7.6)/0.25% phenol red were injected into the cytoplasm of 1- or 2-cell stage embryos. Because of a high mortality rate, pARP-EGFP was injected at a lower concentration (50 μg/ml). Each embryo received 300-500 pl of DNA. The injected embryos were reared in autoclaved Holtfreter's solution (0.35% NaCl, 0.01% KCl and 0.01% $CaCl_2$) supplemented with 1 μg/ml of methylene blue. Expression of GFP was observed and photographed under a ZEISS Axiovert 25 fluorescence microscope.

When zebrafish embryos received pCK-EGFP, GFP expression started about 4 hours after injection, which corresponds to the stage of ~30% epiboly. About 55% of the injected embryos expressed GFP at this stage. The early expression was always in the superficial layer of cells, mimicking endogenous expression of the CK gene as observed by *in situ* hybridization. At later stages, in all GFP-expressing fish, GFP was found predominantly in skin epithelia. A typical GFP transgenic zebrafish fry at 4 days old is shown in Fig. 7.

Under the MCK promoter, no GFP expression was observed in early embryos before muscle cells become differentiated. By 24 hpf, about 12% of surviving embryos expressed GFP strongly in muscle cells and these GFP-positive embryos remain GFP-positive after hatching. The GFP expression was always found in many bundles of muscle fibers, mainly in the mid-trunk region and no expression was ever found in other types of cells. A typical GFP transgenic zebrafish fry (3 days old) is shown in Fig. 8.

Expression of ARP-EGFP was first observed 4 hours after injection at the 30% epiboly stage. The timing of expression is similar to that of pCK-EGFP-injected embryos. However, unlike the CK-EGFP transgenic embryos, the GFP expression under the ARP promoter occurred not only in the superficial layer of cells but also in deep layers of cells. In some batches of injected embryos, almost 100% of the injected embryos expressed initially. At later stages when some embryonic cells become overtly differentiated, it was found that the GFP expression occurred essentially in all different types of cells such as skin epithelia, muscle cells, lens, neural tissues, notochord, circulating blood cells and yolk cells (Fig. 9).

Example IV: Potential applications of fluorescent transgenic fish

The fluorescent transgenic fish have use as ornamental fish in the market. Stably transgenic lines can be developed by breeding a GFP transgenic individual with a wild type fish. By isolation of more zebrafish gene promoters, such as eye specific, bone specific, tail specific etc., and/or by classical breeding of these transgenic zebrafish, more varieties of fluorescent transgenic zebrafish can be produced. Previously, we have reported isolation of over 200 distinct zebrafish cDNA clones homologous to known genes (Gong et al., 1997). These isolated clones code for proteins in a variety of tissues and some of them are inducible by heat-shock, heavy metals, or hormones such as estrogens; thus, this work provided rich resources to isolate tissue-specific and inducible promoters according to the method described in the present invention.

Multiple color fluorescent fish may be generated by the same technique as blue fluorescent protein (BFP) gene, yellow fluorescent protein (YFP) gene and cyan fluorescent protein (CFP) gene are available from Clonetech. For example, a transgenic fish with GFP under an eye specific promoter, BFP under a skin specific promoter, and YFP under a muscle specific promoter will show the following multiple fluorescent colors: green eyes, blue skin and yellow muscle. By recombining different tissue specific promoters and fluorescent protein genes, more varieties of transgenic fish of different fluorescent color patterns will be created. By expression of two or more different fluorescent proteins in the same tissue, an intermediate color may be created. For example, expression of both GFP and BFP under a skin specific promoter, a dark-green skin color may be created.

By using a heavy metal-inducible or hormone- (such as estrogen or other steroid hormone) inducible promoter, a biosensor system may be developed for monitoring environmental pollution. In such a biosensor system, the transgenic fish will glow with a green fluorescence (or other color depending on the fluorescence protein gene used) when pollutants such as heavy metals and estrogens (or their derivatives) reach a threshold concentration in an aquatic environment. Such a biosensor system has obvious advantages over classical analytical methods because it is rapid, visualizable, and capable of identifying specific compounds directly in complex mixture found in an aquatic environment, and is portable or less instrument dependent. Moreover, the biosensor system also provides direct information on biotoxicity and it is biodegradable and regenerative.

In addition, the fluorescent transgenic fish should also be valuable in the market for scientific research tools because they can be used for embryonic studies such as tracing cell lineage and cell migration. Cells from transgenic fish expressing GFP can also be used as cellular and genetic markers in cell transplantation and nuclear transplantation experiments.

The chimeric gene constructs demonstrated successfully in zebrafish in the present invention should also be applicable to other fish species such as medaka, goldfish, carp including koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus (swordtail), hatchet fish, Molly fish, pangasius, etc. The promoters described herein can be used directly in these fish species. Alternatively, the homologous gene promoters can be isolated by the method described in this invention. For example, the isolated and characterized zebrafish cDNA clones and promoters described in this invention can be used as molecular probes to screen for homologous promoters in other fish species by molecular hybridization or by PCR. Alternatively, one can first isolate the zebrafish cDNA and promoters based on the sequences presented in SEQ ID NOS:1, 3, 5

and 7-9 by PCR and then use the zebrafish gene fragments to obtain homologous genes from other fish species by the methods mentioned above.

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    170                 175                 180

ctg gag gga gag ctg aag aac atg cag ggc ctg gtt gag gac ttc aag   689

Leu Glu Gly Glu Leu Lys Asn Met Gln Gly Leu Val Glu Asp Phe Lys
185                 190                 195                 200

aac aag tac gag gat gag atc aac aag cgt gct tcc gta gag aat gag   737

Asn Lys Tyr Glu Asp Glu Ile Asn Lys Arg Ala Ser Val Glu Asn Glu
                205                 210                 215

ttt gtc ctg ctc aag aag gat gtt gat gca gcc tac atg aac aag gtt   785

Phe Val Leu Leu Lys Lys Asp Val Asp Ala Ala Tyr Met Asn Lys Val
            220                 225                 230
```

```
gag ctt gaa gcc aag gtt gat gct ctt cag gat gag atc aac ttc ctc   833

Glu Leu Glu Ala Lys Val Asp Ala Leu Gln Asp Glu Ile Asn Phe Leu
        235                 240                 245

agg gca gtc tac gag gct gaa ctc cgg gag ctc cag tct cag atc aag   881

Arg Ala Val Tyr Glu Ala Glu Leu Arg Glu Leu Gln Ser Gln Ile Lys
    250                 255                 260

gac aca tct gtt gtt gta gaa atg gac aac agc aga aac ctg gat atg   929

Asp Thr Ser Val Val Val Glu Met Asp Asn Ser Arg Asn Leu Asp Met
265                 270                 275                 280

gac tcc atc gtg gct gaa gtt cgc gct cag tat gaa gac atc gcc aac   977

Asp Ser Ile Val Ala Glu Val Arg Ala Gln Tyr Glu Asp Ile Ala Asn
                285                 290                 295

cgc agc cgt gcc gag gca gag agc tgg tac aaa cag aag ttt gag gag   1025

Arg Ser Arg Ala Glu Ala Glu Ser Trp Tyr Lys Gln Lys Phe Glu Glu
            300                 305                 310

atg cag agc acc gct ggt cag tat ggt gat gac ctc cgc tca aca aag   1073

Met Gln Ser Thr Ala Gly Gln Tyr Gly Asp Asp Leu Arg Ser Thr Lys
        315                 320                 325

gct gag att gct gaa ctc aac cgc atg atc gcc cgc ctg cag aac gag   1121

Ala Glu Ile Ala Glu Leu Asn Arg Met Ile Ala Arg Leu Gln Asn Glu
    330                 335                 340

atc gat gct gtc aag gca cag cgt gcc aac ttg gag gct cag att gct   1169

Ile Asp Ala Val Lys Ala Gln Arg Ala Asn Leu Glu Ala Gln Ile Ala
345                 350                 355                 360

gag gct gaa gag cgt gga gag ctg gca gtg aag gat gcc aag ctc cgc   1217

Glu Ala Glu Glu Arg Gly Glu Leu Ala Val Lys Asp Ala Lys Leu Arg
                365                 370                 375

atc agg gag ctg gag gaa gct ctt cag agg gcc aag caa gac atg gcc   1265

Ile Arg Glu Leu Glu Glu Ala Leu Gln Arg Ala Lys Gln Asp Met Ala
            380                 385                 390

cgc cag gtc cgc gag tac cag gag ctc atg aac gtc aaa ttg gct ctg   1313

Arg Gln Val Arg Glu Tyr Gln Glu Leu Met Asn Val Lys Leu Ala Leu
        395                 400                 405

gac att gag atc gcc acc tac agg aaa ctg ttg gaa gga gag gag agc   1361

Asp Ile Glu Ile Ala Thr Tyr Arg Lys Leu Leu Glu Gly Glu Glu Ser
    410                 415                 420
```

aga ctg tcc agc ggt gga gct caa gct acc att cat gtt cag cag acc 1409

Arg Leu Ser Ser Gly Gly Ala Gln Ala Thr Ile His Val Gln Gln Thr
425 430 435 440

tcc gga ggt gtt tca tct ggt tat ggt ggt agc ggc tct ggt ttc ggc 1457

Ser Gly Gly Val Ser Ser Gly Tyr Gly Gly Ser Gly Ser Gly Phe Gly
445 450 455

tac agc agt ggc ttc agc agt ggt ggg tca gga tac ggt agt gga tca 1505

Tyr Ser Ser Gly Phe Ser Ser Gly Gly Ser Gly Tyr Gly Ser Gly Ser
460 465 470

gga ttc ggt tct gga tca ggg tat ggt gga ggc tcc atc agc aaa acc 1553

Gly Phe Gly Ser Gly Ser Gly Tyr Gly Gly Gly Ser Ile Ser Lys Thr
475 480 485

agt gtc acc acc gtc agc agt aaa cgc tat taa ggagaagccc gcccaaaccc 1606

Ser Val Thr Thr Val Ser Ser Lys Arg Tyr
490 495

ccagccgaca cagtttccaa ccttccttac ctgcaactag atcccttctg aaccttctta 1666

cgactcaaac catctatggt gctatatttt agccagacag ctgtcccctg ttaatgagga 1726

gatgtggacg atgattttta aagtacaaaa taagttttag attgttctgt gtgttgatgg 1786

tagttacccg tatcatgcat ctcctgtctg gtggtgtcac tgccatttta aatcatcaac 1846

ccaactacac taaaacgata ccaggaagaa tcgtgctcca agccactgaa tagtcttatt 1906

tctgcactga tatgtacagg gaaagtgaga cacatagaaa ccactgtaac ctacgtagta 1966

ctatggtttc actggatcag gggtgtgcta tacaagttcc tgaatgtctt gtttgaatgt 2026

tttgtgctgt tacaagctcc ctgctgtagt tttgctgact aatctgactt ttgtcatttt 2086

gctatggctg tcagagttgg tttacctatt ttctataaaa tgtatatggc agtcagccaa 2146

taactgatga caattgcttg tgggctacta atgtccagtt acctcacatt caagggagat 2206

ctgttacagc aaaaaacagg cacaatggga tttatgtgga ccatccctcc ttaaccttgt 2266

gtactttccg tgttggaagt ggtgactgta ctgccttaca cattcccctg tattcaactg 2326

gcttccagag catattttac atccccggtt ataaatggaa aatgcaagaa aactgaaaca 2386

atgttcaacc agatttattt ggtattgatt gacgagacac caacttgaaa tttgaataca 2446

ataaatctga gaccacaaaa aaaaaaaaaa aaaa 2480

<210> 2

<211> 498

<212> PRT

<213> Danio rerio

<400> 2

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Met Ser Thr Arg Ser Ile Ser Tyr Ser Ser Gly Gly Ser Ile Arg Arg
 1               5                  10                  15

Gly Tyr Thr Ser Gln Ser Ala Tyr Ala Val Pro Ala Gly Ser Thr Arg
            20                  25                  30

Met Ser Ser Val Thr Ser Val Arg Arg Ser Gly Val Gly Ala Ser Pro
        35                  40                  45

Gly Phe Gly Ala Gly Gly Ser Tyr Ser Phe Ser Ser Ser Ser Met Gly
    50                  55                  60

Gly Gly Tyr Gly Ser Gly Leu Gly Gly Gly Leu Gly Gly Gly Met Gly
65                  70                  75                  80

Phe Arg Cys Gly Leu Pro Ile Thr Ala Val Thr Val Asn Gln Asn Leu
                85                  90                  95

Leu Ala Pro Leu Asn Leu Glu Ile Asp Pro Thr Ile Gln Ala Val Arg
            100                 105                 110

Thr Ser Glu Lys Glu Gln Ile Lys Thr Phe Asn Asn Arg Phe Ala Phe
        115                 120                 125

Leu Ile Asp Lys Val Arg Phe Leu Glu Gln Gln Asn Lys Met Leu Glu
    130                 135                 140

Thr Lys Trp Ser Leu Leu Gln Glu Gln Thr Thr Thr Arg Ser Asn Ile
145                 150                 155                 160

Asp Ala Met Phe Glu Ala Tyr Ile Ser Asn Leu Arg Arg Gln Leu Asp
                165                 170                 175

Gly Leu Gly Asn Glu Lys Met Lys Leu Glu Gly Glu Leu Lys Asn Met
            180                 185                 190

Gln Gly Leu Val Glu Asp Phe Lys Asn Lys Tyr Glu Asp Glu Ile Asn
        195                 200                 205

Lys Arg Ala Ser Val Glu Asn Glu Phe Val Leu Leu Lys Lys Asp Val
    210                 215                 220

Asp Ala Ala Tyr Met Asn Lys Val Glu Leu Glu Ala Lys Val Asp Ala
225                 230                 235                 240

Leu Gln Asp Glu Ile Asn Phe Leu Arg Ala Val Tyr Glu Ala Glu Leu
                245                 250                 255

Arg Glu Leu Gln Ser Gln Ile Lys Asp Thr Ser Val Val Val Glu Met
            260                 265                 270

Asp Asn Ser Arg Asn Leu Asp Met Asp Ser Ile Val Ala Glu Val Arg
        275                 280                 285

Ala Gln Tyr Glu Asp Ile Ala Asn Arg Ser Arg Ala Glu Ala Glu Ser
    290                 295                 300
```

```
Trp Tyr Lys Gln Lys Phe Glu Glu Met Gln Ser Thr Ala Gly Gln Tyr
305                 310                 315                 320

Gly Asp Asp Leu Arg Ser Thr Lys Ala Glu Ile Ala Glu Leu Asn Arg
                325                 330                 335

Met Ile Ala Arg Leu Gln Asn Glu Ile Asp Ala Val Lys Ala Gln Arg
            340                 345                 350

Ala Asn Leu Glu Ala Gln Ile Ala Glu Ala Glu Glu Arg Gly Glu Leu
        355                 360                 365

Ala Val Lys Asp Ala Lys Leu Arg Ile Arg Glu Leu Glu Glu Ala Leu
    370                 375                 380

Gln Arg Ala Lys Gln Asp Met Ala Arg Gln Val Arg Glu Tyr Gln Glu
385                 390                 395                 400

Leu Met Asn Val Lys Leu Ala Leu Asp Ile Glu Ile Ala Thr Tyr Arg
                405                 410                 415

Lys Leu Leu Glu Gly Glu Glu Ser Arg Leu Ser Ser Gly Gly Ala Gln
            420                 425                 430

Ala Thr Ile His Val Gln Gln Thr Ser Gly Gly Val Ser Ser Gly Tyr
        435                 440                 445

Gly Gly Ser Gly Ser Gly Phe Gly Tyr Ser Ser Gly Phe Ser Ser Gly
    450                 455                 460

Gly Ser Gly Tyr Gly Ser Gly Ser Gly Phe Gly Ser Gly Ser Gly Tyr
465                 470                 475                 480

Gly Gly Gly Ser Ile Ser Lys Thr Ser Val Thr Thr Val Ser Ser Lys
                485                 490                 495

Arg Tyr
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<210> 3

<211> 1589

<212> DNA

<213> Danio rerio

<220>

<221> CDS

<222> (86)..(1231)

<220>

<221> primer_bind

<222> (6)..(26)

<223> MCK2

<220>

<221> primer_bind

<222> (20)..(38)

<223> MCK1

<220>

<221> polyA_signal

<222> (1534)..(1539)

<400> 3

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cctatttcgg cttggtgaac aggatctgat cccaaggact gttaccactt ttgttgtctt 60

ttgtgcagtg ttagaaaccg caatc atg cct ttc gga aac acc cac aac aac   112
                              Met Pro Phe Gly Asn Thr His Asn Asn
                                1               5

ttc aag ctg aac tac tca gtt gat gag gag tat cca gac ctt agc aag   160
Phe Lys Leu Asn Tyr Ser Val Asp Glu Glu Tyr Pro Asp Leu Ser Lys
 10                  15                  20                  25

cac aac aac cac atg gcc aag gtg ctg act aag gaa atg tat ggc aag   208
His Asn Asn His Met Ala Lys Val Leu Thr Lys Glu Met Tyr Gly Lys
                 30                  35                  40

ctt agg gac aag cag acc cca cct gga ttc act gtg gat gat gtc atc   256
Leu Arg Asp Lys Gln Thr Pro Pro Gly Phe Thr Val Asp Asp Val Ile
             45                  50                  55

cag act ggt gtt gac aat cca ggc cac ccc ttc atc atg acc gtc ggc   304
Gln Thr Gly Val Asp Asn Pro Gly His Pro Phe Ile Met Thr Val Gly
         60                  65                  70

tgt gtt gct ggt gat gag gag tcc tac gat gtt ttc aag gac ctg ttc   352
Cys Val Ala Gly Asp Glu Glu Ser Tyr Asp Val Phe Lys Asp Leu Phe
     75                  80                  85

gac ccc gtc att tcc gac cgt cac ggt gga tac aag gca act gac aag   400
Asp Pro Val Ile Ser Asp Arg His Gly Gly Tyr Lys Ala Thr Asp Lys
 90                  95                  100                 105

cac aag acc gac ctc aac ttt gag aac ctg aag ggt ggt gat gac ctg   448
```

```
His Lys Thr Asp Leu Asn Phe Glu Asn Leu Lys Gly Gly Asp Asp Leu
                110                 115                 120

gac ccc aac tac ttc ctg agc agc cgt gtg cgt acc gga cgc agc atc   496

Asp Pro Asn Tyr Phe Leu Ser Ser Arg Val Arg Thr Gly Arg Ser Ile
            125                 130                 135

aag gga tac ccc ctg ccc ccc cac aac agc cgt gga gag cgc aga gct   544

Lys Gly Tyr Pro Leu Pro Pro His Asn Ser Arg Gly Glu Arg Arg Ala
        140                 145                 150

gtg gag aag ctg tct gtt gaa gct ctg agt agc ttg gat gga gag ttc   592

Val Glu Lys Leu Ser Val Glu Ala Leu Ser Ser Leu Asp Gly Glu Phe
    155                 160                 165

aag ggc aag tac tac ccc ctg aag tcc atg act gat gac gag cag gag   640

Lys Gly Lys Tyr Tyr Pro Leu Lys Ser Met Thr Asp Asp Glu Gln Glu
170                 175                 180                 185

cag ctg atc gct gac cac ttc ctc ttt gac aaa ccc gtc tcc ccc ctg   688

Gln Leu Ile Ala Asp His Phe Leu Phe Asp Lys Pro Val Ser Pro Leu
                190                 195                 200

ctg ctg gct gct ggt atg gcc cgt gac tgg ccc gat gcc aga ggc att   736

Leu Leu Ala Ala Gly Met Ala Arg Asp Trp Pro Asp Ala Arg Gly Ile
            205                 210                 215

tgg cac aat gag aac aaa gcc ttc ctg gtc tgg gtg aaa cag gag gat   784

Trp His Asn Glu Asn Lys Ala Phe Leu Val Trp Val Lys Gln Glu Asp
        220                 225                 230

cac ctg cgt gtc att tcc atg cag aag ggt ggc aac atg aag gaa gtg   832

His Leu Arg Val Ile Ser Met Gln Lys Gly Gly Asn Met Lys Glu Val
    235                 240                 245

ttc aag cgc ttc tgc gtt ggt ctt cag agg att gag gaa att ttc aag   880

Phe Lys Arg Phe Cys Val Gly Leu Gln Arg Ile Glu Glu Ile Phe Lys
250                 255                 260                 265

aag cac aac cat ggg ttc atg tgg aac gag cat ctt ggt ttc gtc ctg   928

Lys His Asn His Gly Phe Met Trp Asn Glu His Leu Gly Phe Val Leu
                270                 275                 280

acc tgc ccc tcc aac ctg ggc aca ggc ctg cgc ggt gga gtc cac gtc   976

Thr Cys Pro Ser Asn Leu Gly Thr Gly Leu Arg Gly Gly Val His Val
            285                 290                 295

aag ctg ccc aag ctc agc aca cat gcc aag ttt gag gag atc ctg acc   1024
```

```
Lys Leu Pro Lys Leu Ser Thr His Ala Lys Phe Glu Glu Ile Leu Thr
        300                 305                 310

aga ctg cgc ctg cag aag cgt ggc aca ggg ggt gtg gac acc gct tcc   1072

Arg Leu Arg Leu Gln Lys Arg Gly Thr Gly Gly Val Asp Thr Ala Ser
    315                 320                 325

gtt ggt gga gtg ttt gac att tcc aac gct gac cgt atc ggc tct tca   1120

Val Gly Gly Val Phe Asp Ile Ser Asn Ala Asp Arg Ile Gly Ser Ser
330                 335                 340                 345

gag gtt gag cag gtg cag tgt gtg gtt gat ggt gtc aag ctg atg gtg   1168

Glu Val Glu Gln Val Gln Cys Val Val Asp Gly Val Lys Leu Met Val
                350                 355                 360

gag atg gag aag aag ctg gga gaa ggc cag tcc atc gac agc atg atc   1216

Glu Met Glu Lys Lys Leu Gly Glu Gly Gln Ser Ile Asp Ser Met Ile
            365                 370                 375

cct gcc cag aag taa agcgggaggc ccttccattt ttttcttcgt ctttgtctgt   1271

Pro Ala Gln Lys
        380

ttttttacag tccaacagca acgsagagga aaactgctgc tcaaaaagac agtctcacct 1331

ttgcacctgt cttctttcct ttttttccct tcttctctaa tttccatgtc atttcgccat 1391

ctttttttcc actttgtttc ctattaagtc ggtaacatct tgggatcaga tacccggsgc 1451

aggagtgagt gcttgttgct gaggcttcac ctcaatttca gccttggttg taaaaagtga 1511

atcaatcaaa gttgtatttc aaaataaaaa tccccaataa aaaaaaaaaa aaaaaaaaaa 1571

aaaaaaaaaa aaaaaaaa                                              1589
```

<210> 4

<211> 381

<212> PRT

<213> Danio rerio

<400> 4

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Met Pro Phe Gly Asn Thr His Asn Asn Phe Lys Leu Asn Tyr Ser Val
  1               5                  10                  15

Asp Glu Glu Tyr Pro Asp Leu Ser Lys His Asn Asn His Met Ala Lys
             20                  25                  30

Val Leu Thr Lys Glu Met Tyr Gly Lys Leu Arg Asp Lys Gln Thr Pro
         35                  40                  45
```

Pro Gly Phe Thr Val Asp Asp Val Ile Gln Thr Gly Val Asp Asn Pro
50 55 60

Gly His Pro Phe Ile Met Thr Val Gly Cys Val Ala Gly Asp Glu Glu
65 70 75 80

Ser Tyr Asp Val Phe Lys Asp Leu Phe Asp Pro Val Ile Ser Asp Arg
85 90 95

His Gly Gly Tyr Lys Ala Thr Asp Lys His Lys Thr Asp Leu Asn Phe
100 105 110

Glu Asn Leu Lys Gly Gly Asp Asp Leu Asp Pro Asn Tyr Phe Leu Ser
115 120 125

Ser Arg Val Arg Thr Gly Arg Ser Ile Lys Gly Tyr Pro Leu Pro Pro
130 135 140

His Asn Ser Arg Gly Glu Arg Arg Ala Val Glu Lys Leu Ser Val Glu
145 150 155 160

Ala Leu Ser Ser Leu Asp Gly Glu Phe Lys Gly Lys Tyr Tyr Pro Leu
165 170 175

Lys Ser Met Thr Asp Asp Glu Gln Glu Gln Leu Ile Ala Asp His Phe
180 185 190

Leu Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ala Gly Met Ala
195 200 205

Arg Asp Trp Pro Asp Ala Arg Gly Ile Trp His Asn Glu Asn Lys Ala
210 215 220

Phe Leu Val Trp Val Lys Gln Glu Asp His Leu Arg Val Ile Ser Met
225 230 235 240

Gln Lys Gly Gly Asn Met Lys Glu Val Phe Lys Arg Phe Cys Val Gly
245 250 255

Leu Gln Arg Ile Glu Glu Ile Phe Lys Lys His Asn His Gly Phe Met
260 265 270

Trp Asn Glu His Leu Gly Phe Val Leu Thr Cys Pro Ser Asn Leu Gly
275 280 285

Thr Gly Leu Arg Gly Gly Val His Val Lys Leu Pro Lys Leu Ser Thr
290 295 300

His Ala Lys Phe Glu Glu Ile Leu Thr Arg Leu Arg Leu Gln Lys Arg
305 310 315 320

Gly Thr Gly Gly Val Asp Thr Ala Ser Val Gly Gly Val Phe Asp Ile
325 330 335

Ser Asn Ala Asp Arg Ile Gly Ser Ser Glu Val Glu Gln Val Gln Cys
340 345 350

Val Val Asp Gly Val Lys Leu Met Val Glu Met Glu Lys Lys Leu Gly
355 360 365

Glu Gly Gln Ser Ile Asp Ser Met Ile Pro Ala Gln Lys
370 375 380

<210> 5

<211> 1104

<212> DNA

<213> Danio rerio

<220>

<221> CDS

<222> (75)..(1034)

<220>

<221> primer_bind

<222> (45)..(64)

<223> ARP2

<220>

<221> primer_bind

<222> (87)..(112)

<223> ARP1

<220>

<221> polyA_signal

<222> (1069)..(1074)

<400> 5

cgcgtcccta ccgtgagatt ttacaacctt gtctttaaac cggctgttca ccgatccttg 60

gaagcactgc aaag atg ccc agg gaa gac agg gcc acg tgg aag tcc aac 110

Met Pro Arg Glu Asp Arg Ala Thr Trp Lys Ser Asn
1 5 10

tat ttt ctg aaa atc atc caa ctg ctg gat gac ttc ccc aag tgt ttc 158

Tyr Phe Leu Lys Ile Ile Gln Leu Leu Asp Asp Phe Pro Lys Cys Phe
15 20 25

atc gtg ggc gca gac aat gtc ggc tcc aag cag atg cag acc atc cgt 206

```
Ile Val Gly Ala Asp Asn Val Gly Ser Lys Gln Met Gln Thr Ile Arg
     30                  35                  40

ctg tcc ctg cgg ggc aag gcc gtc gtg ctc atg ggg aaa aac acc atg   254

Leu Ser Leu Arg Gly Lys Ala Val Val Leu Met Gly Lys Asn Thr Met
 45                  50                  55                  60

atg agg aag gcc att cgt ggc cac ctg gaa aac aac cca gct ctg gag   302

Met Arg Lys Ala Ile Arg Gly His Leu Glu Asn Asn Pro Ala Leu Glu
                 65                  70                  75

agg ctg ctt ccc cac atc cgc ggg aac gtg ggc ttc gtc ttc acc aag   350

Arg Leu Leu Pro His Ile Arg Gly Asn Val Gly Phe Val Phe Thr Lys
             80                  85                  90

gag gat ctg act gag gtc cga gac ctg ctg ctg gca aac aaa gtg ccc   398

Glu Asp Leu Thr Glu Val Arg Asp Leu Leu Leu Ala Asn Lys Val Pro
         95                 100                 105

gct gct gcc cgt gct ggt gcc atc gcc ccc tgt gag gtg act gtg ccg   446

Ala Ala Ala Arg Ala Gly Ala Ile Ala Pro Cys Glu Val Thr Val Pro
    110                 115                 120

gcc cag aac acc ggg ctc ggt cct gag aag acc tct ttc ttc cag gct   494

Ala Gln Asn Thr Gly Leu Gly Pro Glu Lys Thr Ser Phe Phe Gln Ala
125                 130                 135                 140

ttg gga atc acc acc aag atc tcc aga gga acc att gaa atc ttg agt   542

Leu Gly Ile Thr Thr Lys Ile Ser Arg Gly Thr Ile Glu Ile Leu Ser
                145                 150                 155

gac gtt cag ctt atc aaa cct gga gac aag gtg ggc gcc agc gag gcc   590

Asp Val Gln Leu Ile Lys Pro Gly Asp Lys Val Gly Ala Ser Glu Ala
            160                 165                 170

acg ctg ctg aac atg ctg aac atg ctg aac atc tcg ccc ttc tcc tac   638

Thr Leu Leu Asn Met Leu Asn Met Leu Asn Ile Ser Pro Phe Ser Tyr
        175                 180                 185

ggg ctg atc atc cag cag gtg tat gat aac ggc agt gtc tac agc ccc   686

Gly Leu Ile Ile Gln Gln Val Tyr Asp Asn Gly Ser Val Tyr Ser Pro
    190                 195                 200

gag gtg ctg gac atc act gag gac gcc ctg cac aag agg ttc ctg aag   734

Glu Val Leu Asp Ile Thr Glu Asp Ala Leu His Lys Arg Phe Leu Lys
205                 210                 215                 220

ggt gtg agg aac atc gcc agt gtg tgt ctg cag atc ggc tac cca act   782
```

```
Gly Val Arg Asn Ile Ala Ser Val Cys Leu Gln Ile Gly Tyr Pro Thr
                225                 230                 235

ctt gct tcc atc cct cac act atc atc aat gga tac aag agg gtc ctg   830

Leu Ala Ser Ile Pro His Thr Ile Ile Asn Gly Tyr Lys Arg Val Leu
            240                 245                 250

gct gtc act gtc gaa aca gac tac aca ttc ccc ttg gct gag aag gtg   878

Ala Val Thr Val Glu Thr Asp Tyr Thr Phe Pro Leu Ala Glu Lys Val
        255                 260                 265

aag gcc tac ctg gct gat ccc acc gct ttc gct gtt gca gcc cct gtt   926

Lys Ala Tyr Leu Ala Asp Pro Thr Ala Phe Ala Val Ala Ala Pro Val
    270                 275                 280

gcg gca gct aca gag cag aaa tcc gct gct cct gcg gct aaa gag gag   974

Ala Ala Ala Thr Glu Gln Lys Ser Ala Ala Pro Ala Ala Lys Glu Glu
285                 290                 295                 300

gca ccc aag gag gat tct gag gag tct gat gaa gac atg ggc ttc ggc  1022

Ala Pro Lys Glu Asp Ser Glu Glu Ser Asp Glu Asp Met Gly Phe Gly
                305                 310                 315

ctg ttt gat taa accagacacc gaatatccat gtctgtttaa catcaataaa      1074

Leu Phe Asp
            320

acatctggaa acaaaaaaaa aaaaaaaaaa                                 1104
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<210> 6

<211> 319

<212> PRT

<213> Danio rerio

<400> 6

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Met Pro Arg Glu Asp Arg Ala Thr Trp Lys Ser Asn Tyr Phe Leu Lys
  1               5                  10                  15

Ile Ile Gln Leu Leu Asp Asp Phe Pro Lys Cys Phe Ile Val Gly Ala
            20                  25                  30

Asp Asn Val Gly Ser Lys Gln Met Gln Thr Ile Arg Leu Ser Leu Arg
        35                  40                  45

Gly Lys Ala Val Val Leu Met Gly Lys Asn Thr Met Met Arg Lys Ala
    50                  55                  60

Ile Arg Gly His Leu Glu Asn Asn Pro Ala Leu Glu Arg Leu Leu Pro
65                  70                  75                  80
```

```
His Ile Arg Gly Asn Val Gly Phe Val Phe Thr Lys Glu Asp Leu Thr
                 85                  90                  95

Glu Val Arg Asp Leu Leu Leu Ala Asn Lys Val Pro Ala Ala Ala Arg
            100                 105                 110

Ala Gly Ala Ile Ala Pro Cys Glu Val Thr Val Pro Ala Gln Asn Thr
        115                 120                 125

Gly Leu Gly Pro Glu Lys Thr Ser Phe Phe Gln Ala Leu Gly Ile Thr
    130                 135                 140

Thr Lys Ile Ser Arg Gly Thr Ile Glu Ile Leu Ser Asp Val Gln Leu
145                 150                 155                 160

Ile Lys Pro Gly Asp Lys Val Gly Ala Ser Glu Ala Thr Leu Leu Asn
                165                 170                 175

Met Leu Asn Met Leu Asn Ile Ser Pro Phe Ser Tyr Gly Leu Ile Ile
            180                 185                 190

Gln Gln Val Tyr Asp Asn Gly Ser Val Tyr Ser Pro Glu Val Leu Asp
        195                 200                 205

Ile Thr Glu Asp Ala Leu His Lys Arg Phe Leu Lys Gly Val Arg Asn
    210                 215                 220

Ile Ala Ser Val Cys Leu Gln Ile Gly Tyr Pro Thr Leu Ala Ser Ile
225                 230                 235                 240

Pro His Thr Ile Ile Asn Gly Tyr Lys Arg Val Leu Ala Val Thr Val
                245                 250                 255

Glu Thr Asp Tyr Thr Phe Pro Leu Ala Glu Lys Val Lys Ala Tyr Leu
            260                 265                 270

Ala Asp Pro Thr Ala Phe Ala Val Ala Ala Pro Val Ala Ala Ala Thr
        275                 280                 285

Glu Gln Lys Ser Ala Ala Pro Ala Ala Lys Glu Glu Ala Pro Lys Glu
    290                 295                 300

Asp Ser Glu Glu Ser Asp Glu Asp Met Gly Phe Gly Leu Phe Asp
305                 310                 315
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<210> 7

<211> 2241

<212> DNA

<213> Danio rerio

<220>

<221> TATA_signal

<222> (2103)..(2108)

<220>

<221> primer_bind

<222> (2221)..(2241)

<223> CK2

<220>

<221> misc_feature

<222> (2142)..(2235)

<223> Identical to the 5' CK cDNA

<400> 7

ccttcccttc tacttttgac gtccttttaa gagcttgtgc atgaaagcag atttggagct 60

gattactcat ctcaaacacc catacaaagg gatgattgcc gtaccatgat ctcacacctt 120

tcacacctgg tttatactat gatagttgta gacgattgcg taatgctatt aaatgcccat 180

cagtgctggc tgtgacaccc aactgctgcc atttcgtgtt gacttgcacg agaaatgaga 240

aattgtctga ctatgcaggg tgtctatgcg tgggaacatt tatcagtggt cattaaatac 300

tatagtttac agttagacca aagtgtgctg tatttttgtg ttagcttagc tgcagttttt 360

gtgtgtgaag taacaaatga caaatactca aactattgta attaagtagt ttttctcaga 420

aattgtaatt tactaagtag tttaaaaatg tgtactttta ctttcccttg agtacatttt 480

tagtgcagtg ttggtacttt tatttcactt ccttccttca acctgcagtc actactttat 540

ttattcttgt ctatgtggat tagacaaatc agtcctgtga ttcctgtcca atcaaattgc 600

acatagaagg taaatcacat cataatgaac taccttaaga catgggccat ttataattgc 660

agcaaactgt ttgccagcat taaaagaaga tgtcaaaaat atttacacgc attaacccag 720

agactgctta gatgcatgtc actgatgaga agatgatgga tgtttactgt atgatgaccg 780

aaataacttt aaacgcacac aagacggcac aagacgtcaa catggcgtta ggttgacgtt 840

gtaccccaac gcagtgggga cgttgcattt tgtttagaaa tgaaaattag gttgacgtca 900

gaactcaacg tcaggtcgat gtcaatgttc aacatccaat ctaaaatcat atatcaatgt 960

ctaatgatgt tacagcttga tgttatgcgg atgttacccc tatgacgtct atcagacgtt 1020

ggattatggt tgccatacct gatgaataaa tgtcattatt tgacgttggt ttaagatgtt 1080

ggttcgacat tggattttgg tcgctttcca acacaaccta aatccaccaa atattaactt 1140

cctatgacat cgttattgga cgtcaaaata acaatatcct tagatgctgg ctagactttg 1200

aatttaggtc accacaacct atatttaacc taatattaac atcttatgat gttgtgtgcc 1260

tgctgggcaa taactaaatg cactacagaa tgttacgttt acacacatgt aaattacatg 1320

taaatgcatc agcttttcac agcataatac tcactactta ctactcttga gtacttttaa 1380

aaaagctact tttcactcat actttgagta atatttacaa ctgatacttt tactcgcact 1440

acatttttag gcatgtattg atatttttac tatgattttt cagtactctt tccactactg 1500

cagccctccc catacataat cgtatgttta cacatatggt ggagtttaga gccataatct 1560

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atatcaatac agaaggaaac tgtgttccct gttcccttgt aaatctcaac aatgcaactg 1680

ttcagctcag ggggaaaaat gccctgccag atccaaacgg ctggcaaaag tgaatggaaa 1740

aaagcctttc attaatgtga aagttgctgc gcgccccacc cagataaaaa gagcagaggt 1800

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ctcaagcctc tccttgcatt ctctcattcc acctggggca aagccccagg ctgggtgtga 1980

caacatttat cttaccactt tctctctgta cctgtctaac aggtagggtg tgtgtgagag 2040

tgcgtatgtg tgcaagtgcg tgtgtgtgtg agagcagtca gctccaccct ctcaagagtg 2100

tgtataaaat tggtcagcca gctgctgaga gacacgcaga gggactttga ctctcctttg 2160

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actcaaagac acaggatccg g 2241

<210> 8

<211> 1456

<212> DNA

<213> Danio rerio

<220>

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<220>

<221> primer_bind

<222> (1433)..(1456)

<223> MCK2

<220>

<221> misc_feature

<222> (1428)..(1453)

<223> Identical to the 5' MCK cDNA

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tgtggcttaa tcttggctga tgtgtgtgtg tgtgtgtgtg tacttgacag ctgctagtga 120

gcatgtgcac catgacaggc ctgttattca cacttggtgc catgttggag actgttcggc 180

cagctatagt tttcttcaca gagtcctggg tcacctaatg tcacaaggaa gaaacatgtt 240

acatgttaaa atgtgacatt caaattgtag tgcattactt aacgaaacgc attacacaag 300

ttacagctta aaagattgct agacagaaaa accagggagg ggttttccca taatatccag 360

tgagactcta ggagcgggaa cactaacagg cctccctgag tgagaacatt gcatgtgcgc 420

gtgacagaaa accagagatg gaaatacctt cttttgaatt gcataattgc ttaaaagaag 480

acacaacagg gatagttcac ccaaaaaaca gaccattctt tttttctgtt gaacaaaaat 540

taagatattt tgaagaatgc ttaccgaata acttccatat ttggaaacta attacagtga 600

aagtcaatgg gtcttccagc atttttttcaa tataccttac tttgagttca aaagaaaaac 660

acatctcaaa taggtttgag gttgaataaa cattttttcat tttggggtgg actatcccta 720

attatttgac acttaagatt tatagtaaat cattttatag actttctccc cttattaaac 780

atggttgaat ttatcttcat gtttatgtct gggttgtgct tttttgaaaa gatttccctg 840

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gcgcacttct gatatctgtg gtatgtttgg caaaagtgct gctcagcctt tttagcatgg 1140

cagatcctcc acatcccatc acccctcctt caacctattc cctcctggaa agctatgtat 1200

ggggcgggaa gtgtaaatgg atatgggaag gaaggggggc accacccaca gctgccacct 1260

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ccagcgccag ccaatcataa ttcagtgagc tctaaaatgg gccagccaat ggctgcaggg 1380

gctagaggta tatatatcca aatcaaactc ttcttgcttg ggtgacccct atttcggctt 1440

ggtgaacagg atccgg 1456

<210> 9

<211> 2205

<212> DNA

<213> Danio rerio

<220>

<221> primer_bind

<222> (2179)..(2205)

<223> ARP2

<220>

<221> misc_feature

<222> (2153)..(2199)

<223> Identical to the 5' ARP cDNA

<220>

<221> intron

<222> (792)..(2152)

<220>

<221> misc_feature

<222> (775)..(791)

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agaaataata caaatttgtt tacagtattc ttagttattg caataaacga attttatata 180

gaaagagaaa gagttttatt ataagatgtt caatttaaaa aatggcagaa aatagaaaaa 240

tgattgtcaa gatgataaaa gtcagtttag acaaaaaaat aagatgaaaa acatcaaaat 300

agataataaa gtgacttttt tgggcggacc aaatttccct attaatggtc aattcattaa 360

aatacattca ttaaaataaa ggtattgcga tgaatttaga tgcacagtga ttttggttct 420

gtgcagattt ttggctgttg ttagaaggga tacatctgcg gccgaaagtt aacgggaact 480

atttacattc tttgctatta aattatccat tatttgtatt ttattacccc aaccgtaaac 540

tcaaccctca cagtaatgta aaaatattat ttattgtttt atagcgtcac agaatgatgc 600

tatattgacc gcagctgtat cctttctaag tgcgactgta caaatacgca ctgaccgtga 660

cagacacgtg cattgaccaa tcagcgcaca gatacgcatt ttccgcgcga ttctgattgg 720

atgatcgact gatactaata ttgtgccgct tcctttcgcg gcctctttct ttcacgcgtc 780

cctaccgtga ggtaaggctg acgccgctct tgtggcggtt tcttaaaatg tgttaataaa 840

taacatcata agaggtcacg agaaggtcta cgtgtgttta atatcagcgg cggttattat 900

tatgcgttta aagcttgtgt aatgattttt acagtaaaag ttagcactag cctgttagca 960

caggcctcgt gcgccatgtg tgacgcgacg ttttaatagc atcttatttg attttgatga 1020

tccgattctg atattaatca tatttatgcg taaaatgtgt gatgggtctg ctagtggaca 1080

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tcaatcacat ataattgtgt ttatgtttta tttgagtcat cataccaggt aatagtttta 1260

tttatattag tatgtacaat ttggcataaa ctgccttcgg ttttgattga catctacttt 1320

gtaaagttaa tcttaaaggg gtaaaggctc acccaaaaga caattcaccg tcaagtgttt 1380

tcaaatctta tgagtttctt aatgaacatg gtatgttttg gagaaaactg gaaaccaact 1440

accataatac aaatacagga aaaatatact atagaagtcg atggttacag gttttctgca 1500

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tgcataaatc agttttcatt tgggtgagct gtctctaaac atttgattta gacacctcag 1620

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atagcagtaa atcaaataca atagtgctct gattatcttt aaatatttga aagcttataa 2040

taggcaacca aattacctgg aaacagttta caaacagtaa ttcatatttt gtcatttaat 2100

aagatgcaca caaggcaggt gtaaaagtat tgcttgtgtt tgtaatcctc agattttaca 2160

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<210> 10

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cytokeratin gene specific primer

<400> 10

cgctggagta agagatagac ctgg 24

<210> 11

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cytokeratin gene specific primer

<220>

<221> misc_feature

<222> (1)..(6)

<223> Introduced for restriction site

<220>

<221> misc_feature

<222> (3)..(8)

<223> BamHI site

<400> 11

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<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Muscle creatine kinase gene specific primer

<220>

<221> misc_feature

<222> (3)..(8)

<223> BamHI site

<400> 12

ccggatcctt gggatcagat cctg 24

<210> 13

<211> 24

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Muscle creatine kinase gene specific primer

<220>

<221> misc_feature

<222> (1)..(3)

<223> Introduced for restriction site

<220>

<221> misc_feature

<222> (3)..(8)

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<210> 14

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Acidic ribosomal protein PO gene specific primer

<400> 14

tagttggact tccacgtgcc ctgtc 25

<210> 15

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Acidic ribosomal protein PO gene specific primer

<220>

<221> misc_feature

<222> (1)..(7)

<223> Introduced for restriction site

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<221> misc_feature

<222> (1)..(6)

<223> BamHI site

<400> 15

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<210> 16

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide for linker used in linker-mediated PCR

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<210> 17

<211> 10

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide for linker used in linker-mediated PCR

<220>

<223> n is a dideoxycytidine

<400> 17

gaattcaagn 10

<210> 18

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: linker specific primer

<400> 18

gttcatcttt acaagctagc g 21

<210> 19

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: linker specific primer

<400> 19

tcctgaacaa tgctgtggac 20

CLAIMS

1. A zebrafish cytokeratin gene promoter which is capable of directing a structural gene to be predominantly expressed in skin epithelia when it is inserted in front of the structural gene and introduced into fish embryos.

2. A zebrafish muscle creatine kinase gene promoter which is capable of directing a structural gene to be specifically expressed in muscles when it is inserted in front of the structural gene and introduced into fish embryos.

3. A zebrafish acidic ribosomal protein P0 gene promoter which is capable of directing a structural gene to be expressed ubiquitously in all tissues when it is inserted in front of the structural gene and introduced into fish embryos.

4. A recombinant DNA molecule comprising a structural gene and the promoter of claim 1, 2 or 3 arranged upstream of said promoter.

5. A chimeric gene comprising the promoter of claim 1, 2 or 3, operatively linked to DNA encoding a protein selected from the group consisting of GFP, modified GFP, EGFP, BFP, EBFP, YFP, EYFP, CFP, ECFP, luciferase, ß-galactosidase, and chloramphenicol acetyltransferase.

6. A transgenic fish comprising a chimeric gene comprising the promoter of claim 1, 2 or 3.

7. The transgenic fish of claim 6, which contains said promoter in germ cells and/or in somatic cells and which is capable of breeding with either a said transgenic fish or a non-transgenic fish to produce viable and fertile transgenic progeny.

8. The transgenic fish of claim 6, and progeny of said fish that emits green fluorescence under a blue light.

9. A transgenic fish comprising a DNA that encodes a fluorescent protein under control of a promoter that causes said DNA (1) to be expressed in predominately skin epithelia, (2) to be specifically expressed in muscles or (3) to be expressed ubiquitously in all tissues.

10. The transgenic fish of claim 9, wherein said promoter is a promoter which naturally occurs in non-transgenic fish of the same species as the transgenic fish.

11. A recombinant DNA vector comprising a promoter DNA that hybridizes under stringent conditions to a polynucleotide of any one of SEQ ID NOS:7, 8 or 9, operatively linked to a structural gene encoding a fluorescent or chemiluminescent protein.

12. A cell transformed with the vector of claim 11.

13. A transgenic fish comprising a chimeric gene in turn comprising a promoter DNA that hybridizes under stringent conditions to a polynucleotide of any one of SEQ ID NOS:7, 8 or 9, operatively linked to a structural gene encoding a fluorescent or a chemiluminescent protein.

14. A method for sensing a steroid hormone or a steroid hormone derivative in a water sample comprising:

(a) contacting a fish expressing a fluorescent or chemiluminescent protein under control of an estrogen- or other steroid hormone-inducible promoter with a sample of water; and

(b) measuring the amount of fluorescent or chemiluminescent light from said fish.

ABSTRACT

CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

Three zebrafish gene promoters, which are skin specific, muscle specific and ubiquitously expressed respectively, were isolated and ligated to the 5' end of the EGFP gene. When the resulting chimeric gene constructs were introduced into zebrafish, the transgenic zebrafish emit green fluorescence under a blue light according to the specificity of the promoters used. Thus, new varieties of ornamental fish of different fluorescence patterns, e.g., skin fluorescence, muscle fluorescence, and/or ubiquitous fluorescence, are developed.

(FIG. 4 is to be published)

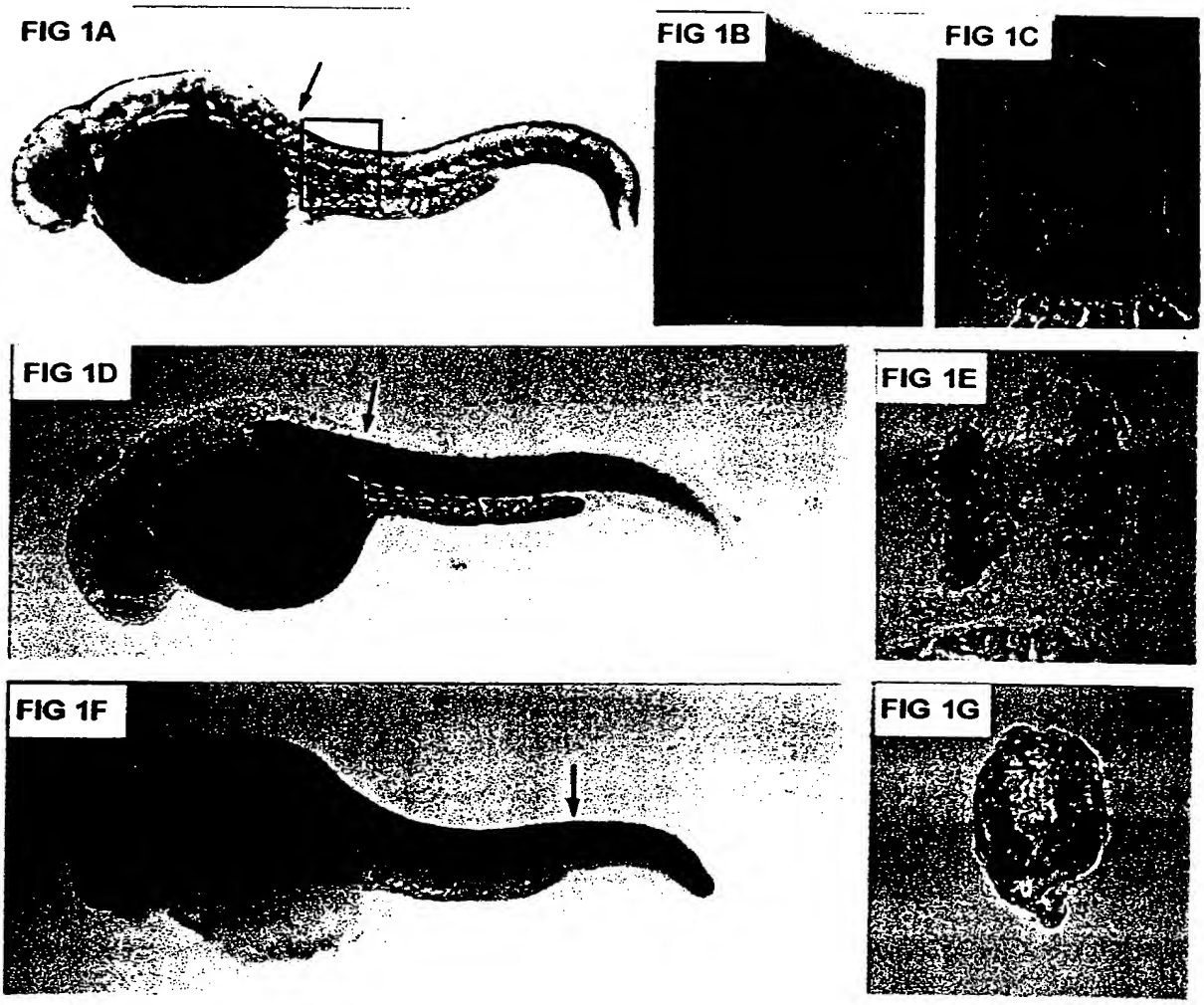

FIG. 1

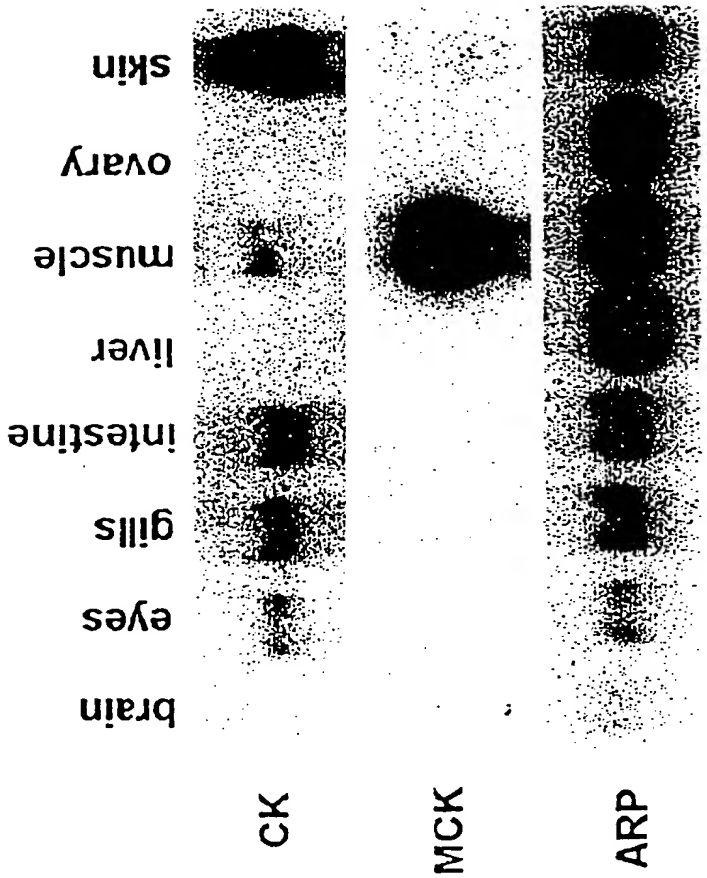
CK
MCK
ARP
brain
eyes
gills
intestine
liver
muscle
ovary
skin

FIG. 2

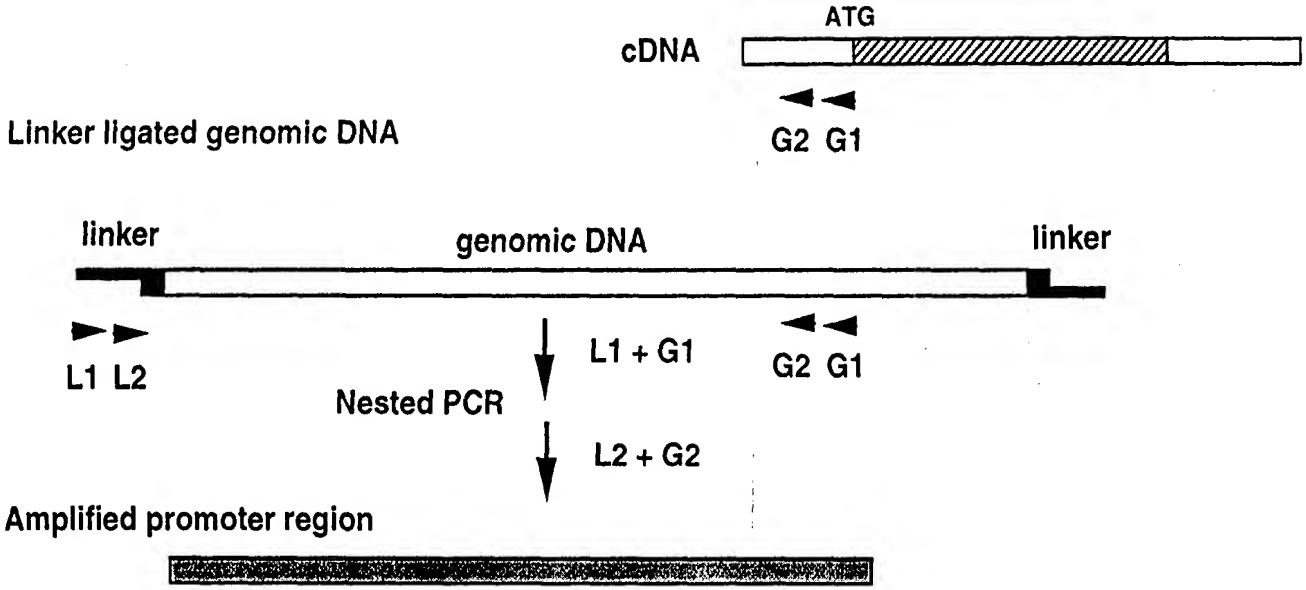
ATG
cDNA
G2 G1
Linker ligated genomic DNA
linker
genomic DNA
linker
L1 L2
L1 + G1
G2 G1
Nested PCR
L2 + G2
Amplified promoter region

FIG. 3

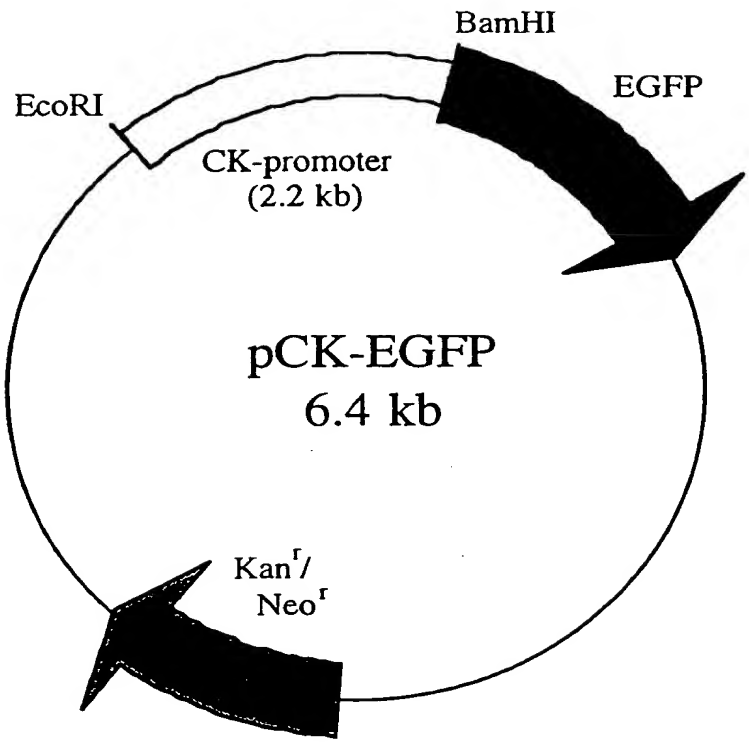

FIG. 4

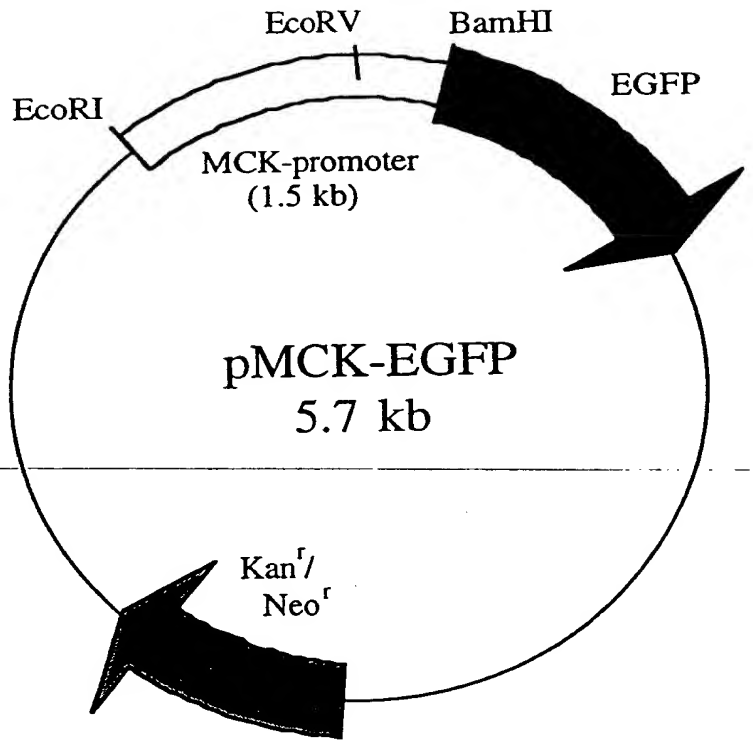
EcoRV
BamHI
EGFP
EcoRI
MCK-promoter
(1.5 kb)
pMCK-EGFP
5.7 kb
Kan^r/
Neo^r

FIG. 5

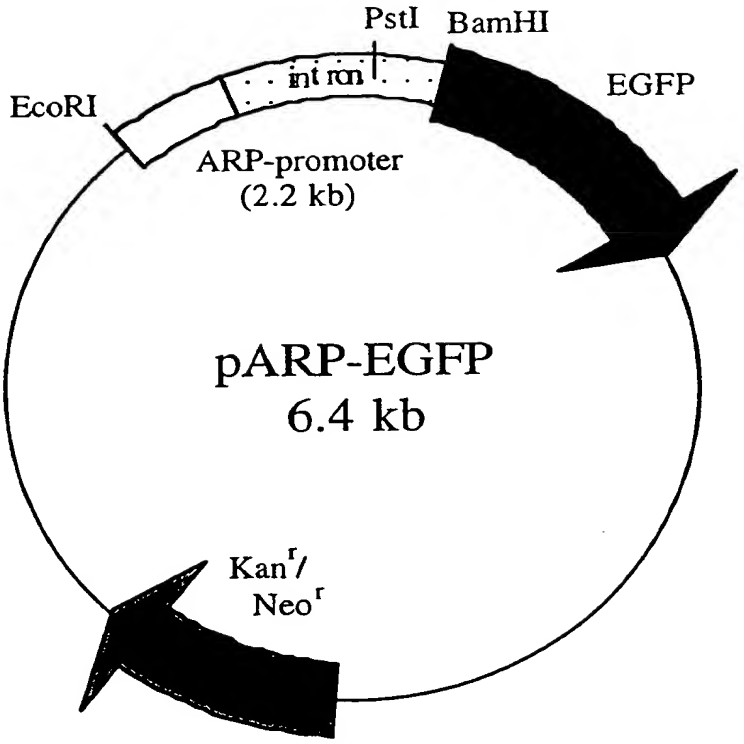
PstI
BamHI
EGFP
EcoRI
intron
ARP-promoter
(2.2 kb)
pARP-EGFP
6.4 kb
Kan^r/
Neo^r

FIG. 6

FIG. 7

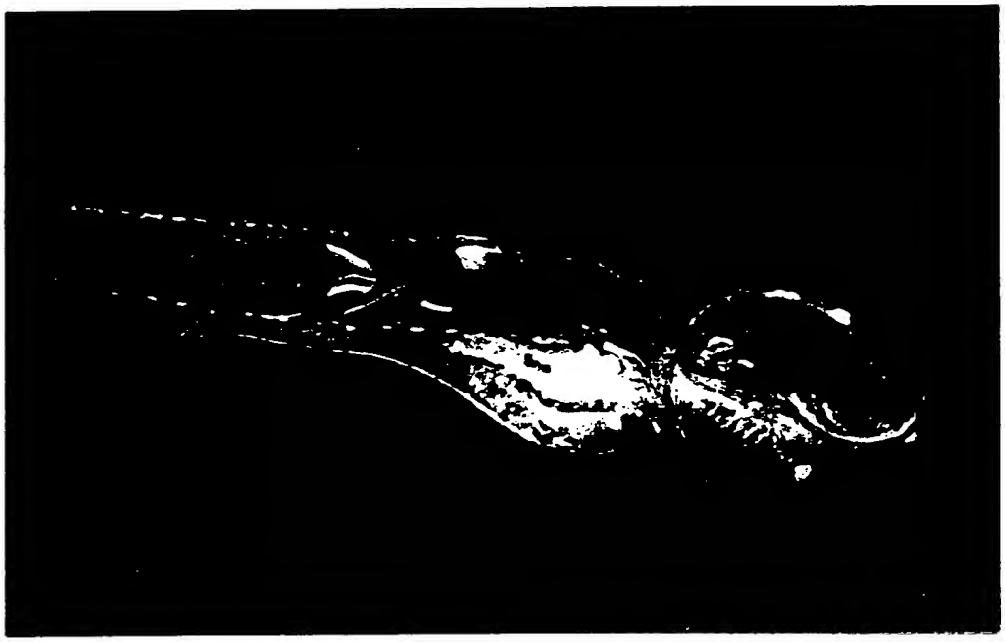

FIG. 8

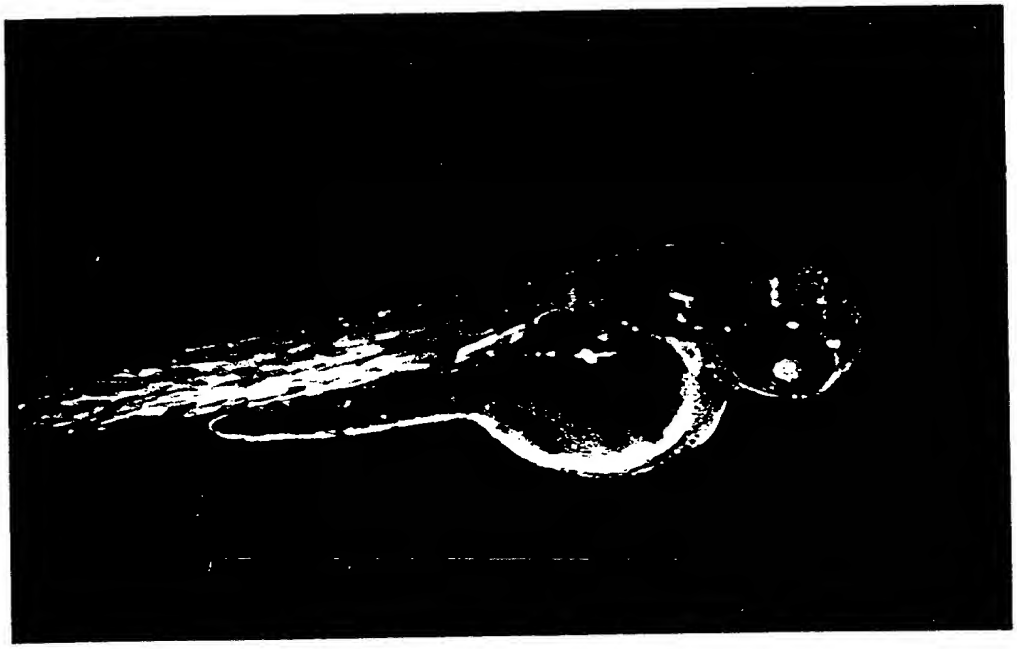

FIG. 9